Functional imaging of legumain in cancer using a novel quenched activity-based probe

Laura E. Edgington[1,2], Martijn Verdoes[2], Alberto Ortega[2], Jiyoun Lee[2, †], Salahuddin Syed[2], Michael H. Bachmann[3], Galia Blum[2, †] and Matthew Bogyo[2,6,*]

[1]Cancer Biology Program, [2]Departments of Pathology [3]Pediatrics, and [4]Microbiology and Immunology, Stanford School of Medicine, 300 Pasteur Dr. Stanford, California, 94305-5324.

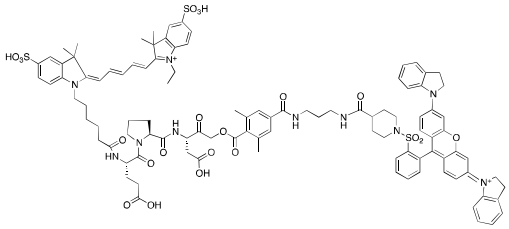
Supporting Information

Synthesis Methods

All resin and reageants were puchased from commercial suppliers and used without further purification. All solvents used were HPLC grade. Water-sensitive reactions were performed in anhydrous solvents under positive pressure of argon. Reactions were analyzed by LC-MS using an API 150EX single-quadropole mass spectrometer (Applied Biosystems). Reverse HPLC was conducted with an AKTA explorer 100 (Amersham Pharmacia Biotech) using a C18 column. ESI-MS was performed on an Agilent 1260 HPLC and Bruker MicroTOF-Q II by the Stanford Mass Spec Facility. ^{1}H NMR spectrum was obtained using a Varian Inova 600/125 MHz) equipped with a pulsed field gradient assessory.

This method was adapted from a previously described literature procedure (Blum et al. 2005, 2007). Boc-Glu(OtBu)-Pro-Asp-(OtBu)-OH was prepared using standard solid phase peptide synthesis on 2-chlorotrityl resin. This acid was converted to a chloromethylketone using the previously described method. To a solution of Boc-Glu(OtBu)-Pro-Asp-(OtBu)-OH (188mg, 0.33 mmol) and N-methylmorpholine (44.5 µl, 0.41 mmol) in THF (2 mL) at -78°C was added isobutylchloroformate (49.5 µl, 0.38 mmol). Diazomethane was prepared from diazald (0.4g, 0.41mmol) and added dropwise to the reaction mixture at 0°C. The reaction proceeded for 30 minutes at 0°C and then warmed to room temperature for three hours. Then a 1:1 solution of acetic acid and hydrochloric acid (1 mL) was added dropwise while stirring at 0°C . The reaction mixture was then diluted with EtOAc and washed with water, saturated $NaHCO_3$, and brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated. A yellow oil was obtained (180 mg). The crude material was then used without further purification. Meanwhile, Fmoc-diaminopropane was added to chlorotrityl resin (0.075 mmol) followed by standard amide coupling with an excess of 2,6-dimethylterephthalic acid. Boc-Glu(OtBu)-Pro-Asp-(OtBu)-CMK (45 mg, 0.074 mmol) was then added to the resin with 20 equivalents of KF and rocked overnight. Compound was cleaved from the resin, concentrated using toluene, and then purified by HPLC. A white powder was obtained (14 mg, 0.017 mmol, 23%). QSY21-OSu was then coupled by published methods, followed by HPLC purification (60%). Boc and OtBu groups were removed in 25% TFA in DCM and then the compound was concentrated under vacuum. Lastly, Cy5-OSu was coupled by published methods followed by HPLC purification (60%). This yielded the final compound, **LE28**, a blue powder.

Blum, G.; Mullins, S. R.; Keren, K.; Fonovic, M.; Jedeszko, C.; Rice, M. J.; Sloane, B. F.; Bogyo, M. *Nat Chem Biol* **2005**, *1*, 203.
Blum, G.; von Degenfeld, G.; Merchant, M. J.; Blau, H. M.; Bogyo, M. *Nat Chem Biol* **2007**, *3*, 668.

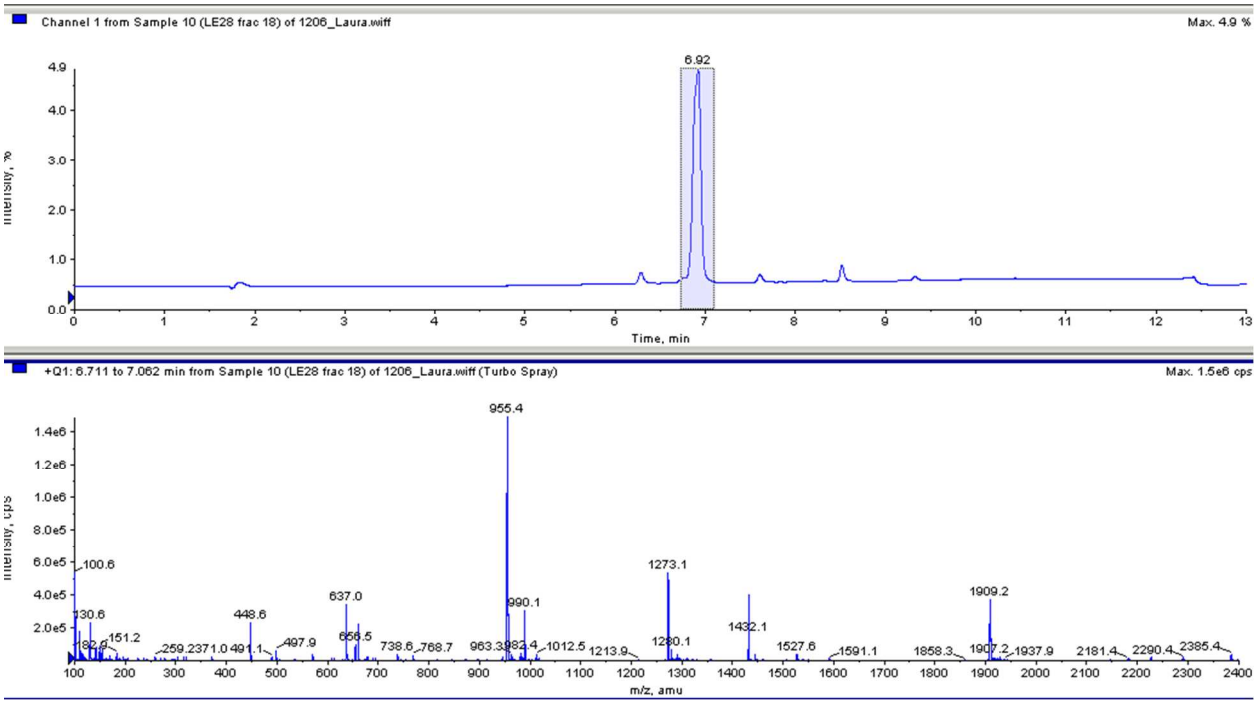


LE28
Chemical Formula: $C_{102}H_{112}N_{10}O_{21}S_3^{2+}$
Exact Mass: 1908.72
Molecular Weight: 1910.23

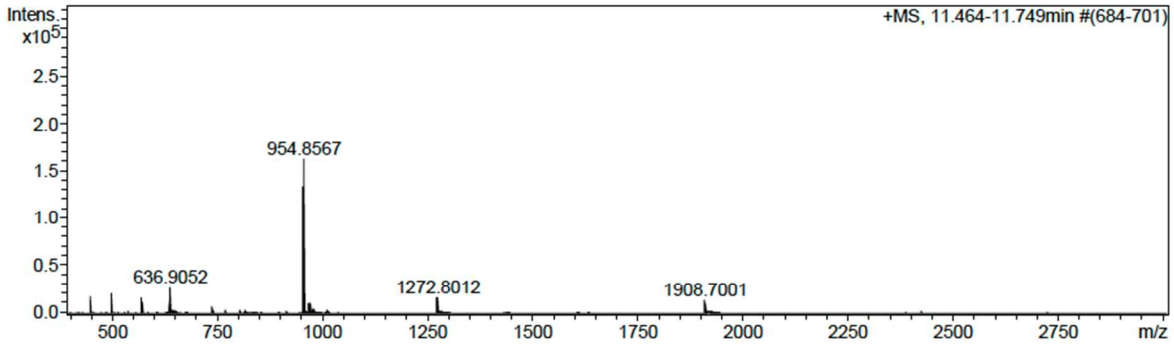
Purity assessed by LCMS, shown below.
HRMS: m/z calculated for $[C_{102}H_{112}N_{10}O_{21}S_3]^{2+}$ is 954.3577, found 954.3544

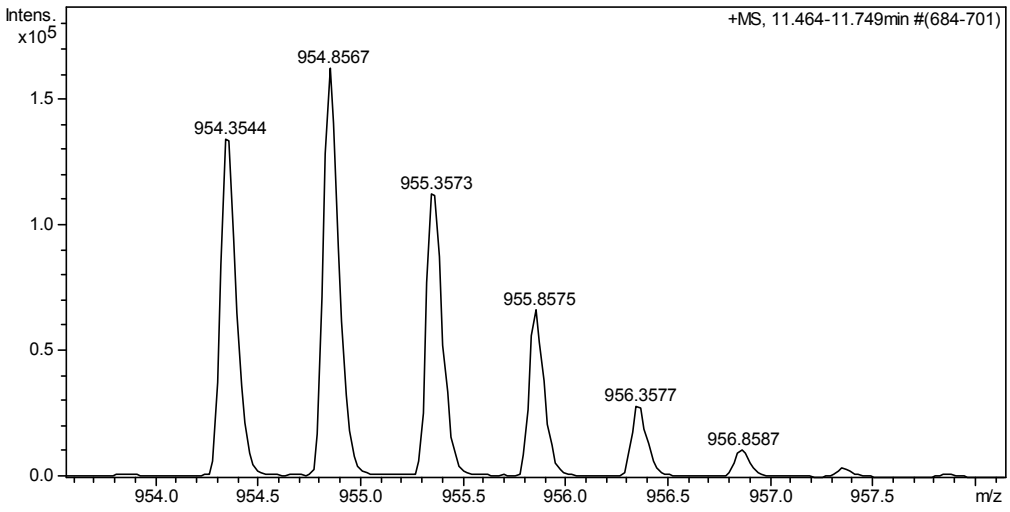
Figure S1 (below) (A) LCMS trace (B) HRMS spectrum (C) ^{1}H NMR spectrum LE28

A.

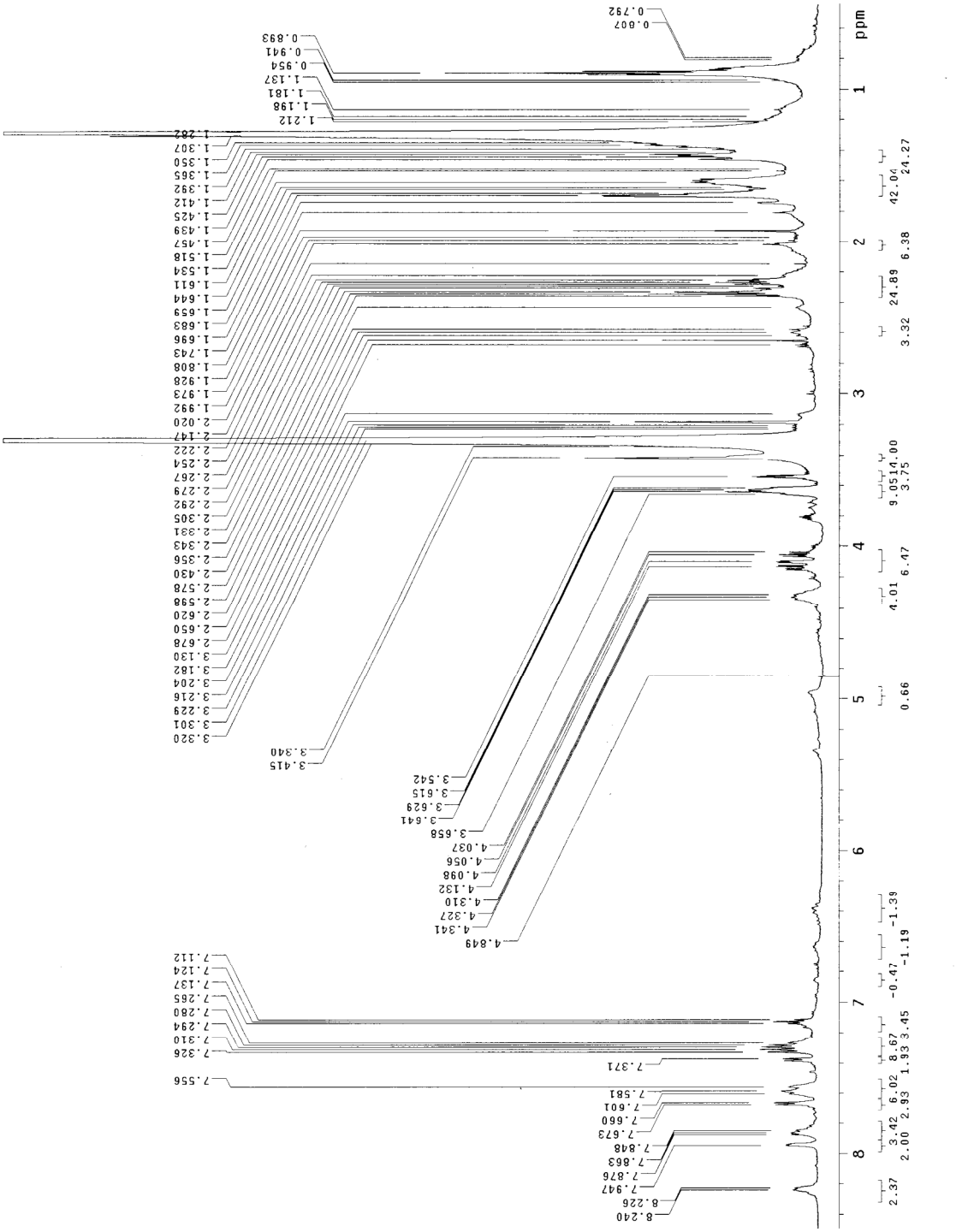

Channel 1 from Sample 10 (LE28 frac 18) of 1206_Laura.wiff
Max. 4.9 %
6.92
Intensity, %
Time, min
+Q1: 6.711 to 7.062 min from Sample 10 (LE28 frac 18) of 1206_Laura.wiff (Turbo Spray)
Max. 1.5e6 cps
955.4
1273.1
990.1
637.0
448.6
100.6
130.6
151.2
1909.2
1432.1
1280.1
656.5
497.9
1527.6
1591.1
1858.3
1907.2
1937.9
2181.4
2290.4
2385.4
Intensity, cps
m/z, amu

B.


Intens.
x10^5
+MS, 11.464-11.749min #(684-701)
954.8567
636.9052
1272.8012
1908.7001
m/z


Intens.
x10^5
+MS, 11.464-11.749min #(684-701)
954.8567
954.3544
955.3573
955.8575
956.3577
956.8587
m/z

C.



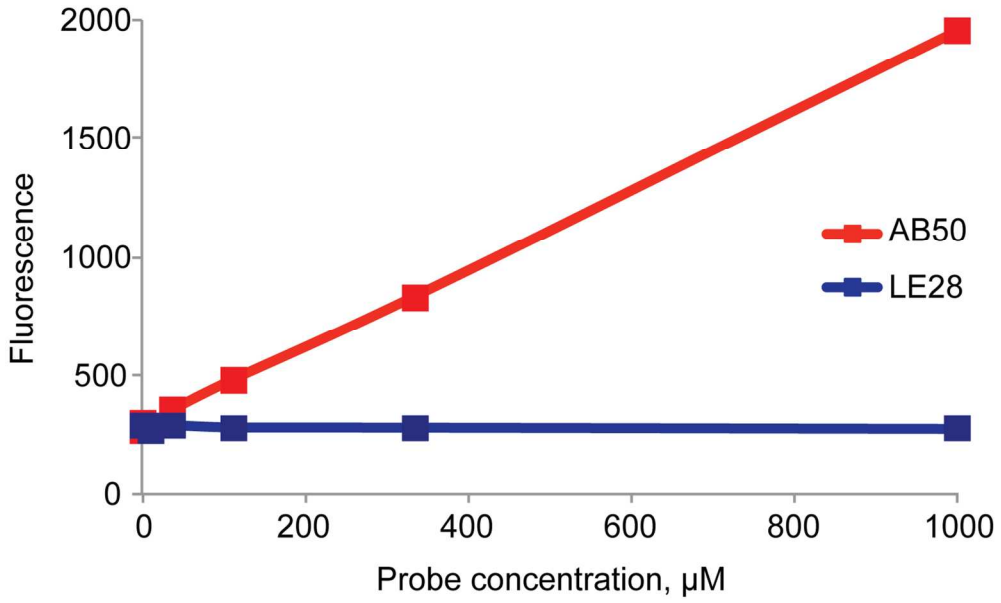


Figure S2. Quenching efficiency. LE28 and AB50 were diluted serially in 1% DMSO in a clear bottom 96-well plate. Fluorescence was measured using a spectrophotometer at 633 nm/670 nm excitation/emission and then plotted as a function of concentration.

Figure S3. The following charts contain data from BCA protein quantification assays that were performed before each SDS-PAGE experiment to ensure equal loading.

Figure 2A, 2B, S3A, S5A were all performed using the same batch of RAW cell lysates in citrate buffer. For each sample, 5.5 µl of lysate (5.5 µg/µl) were diluted into 24.5 µl buffer to yield a final concentration of 1µg/µl (30 µg total)

LE28 dose curve in RAW cells
Figure 2C

BSA Standard	
mg/ml	**A562**
2	1.0336
1	0.5693
0.75	0.4341
0.5	0.3088
0.25	0.1782
0.125	0.0875
slope	0.49
y-intercept	0.05
R2	0.997

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
1	0.2444	0.39	3.88	2.91	17.19
2	0.2988	0.50	4.97	3.73	13.41
3	0.2672	0.43	4.34	3.25	15.37
4	0.2591	0.42	4.17	3.13	15.97
5	0.2484	0.40	3.96	2.97	16.84
6	0.2312	0.36	3.61	2.71	18.45
7	0.2614	0.42	4.22	3.16	15.80

LE28 time course in RAW cells
Figure 2D

BSA Standard	
mg/ml	**A562**
2	0.6922
1	0.3526
0.75	0.302
0.5	0.1743
0.25	0.0483
0.125	0.1127
slope	0.35
y-intercept	0.006
R2	0.986

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
1	0.3514	1.01	10.05	7.54	6.63
2	0.3601	1.03	10.30	7.72	6.47
3	0.3878	1.11	11.07	8.31	6.02
4	0.445	1.27	12.68	9.51	5.26
5	0.4044	1.15	11.54	8.66	5.78
6	0.4922	1.40	14.00	10.50	4.76
7	0.468	1.33	13.33	9.99	5.00
8	0.4675	1.33	13.31	9.98	5.01

Experiment RAW cells treated with DMSO or LI-1
Figure 2G

BSA Standard	
mg/ml	**A562**
2	0.8396
1.5	0.7009
1	0.548
0.75	0.442
0.5	0.33
0.125	0.0959
slope	0.45
y-intercept	0.04
R2	0.978

			Loaded (□l)
Sample	**A562**	**mg/ml**	**50 µg**
DMSO	0.2921	4.80	10.42
LI-1	0.3128	5.33	9.39

Macrophage Cytokine Activation
Figures 3A-D and S6A-C

BSA Standard	
mg/ml	**A562**
2	0.7673
1	0.4341
0.75	0.3271
0.5	0.222
0.25	0.1152
0.125	0.0529
slope	0.38
y-intercept	0.02
R2	0.994

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:5**	**x 0.75**	**50 µg**
1	0.3296	0.80	3.98	2.99	16.73
2	0.3165	0.76	3.81	2.86	17.49
3	0.3241	0.78	3.91	2.93	17.04
4	0.3409	0.83	4.13	3.10	16.13
5	0.352	0.86	4.28	3.21	15.58
6	0.3386	0.82	4.10	3.08	16.25
7	0.295	0.71	3.53	2.65	18.90
8	0.3131	0.75	3.77	2.82	17.70
9	0.2994	0.72	3.59	2.69	18.59
10	0.3358	0.81	4.07	3.05	16.40
11	0.3192	0.77	3.85	2.89	17.33
12	0.3083	0.74	3.70	2.78	18.00
13	0.3143	0.76	3.78	2.84	17.63
14	0.3295	0.80	3.98	2.99	16.74
15	0.2823	0.67	3.36	2.52	19.84
16	0.3114	0.75	3.74	2.81	17.81
17	0.3426	0.83	4.16	3.12	16.04
18	0.3463	0.84	4.20	3.15	15.86
19	0.3172	0.76	3.82	2.87	17.45
20	0.3312	0.80	4.00	3.00	16.65

21	0.3369	0.82	4.08	3.06	16.34
22	0.3551	0.86	4.32	3.24	15.43
23	0.3631	0.89	4.43	3.32	15.06
24	0.3697	0.90	4.51	3.38	14.77
25	0.3743	0.91	4.57	3.43	14.58
26	0.371	0.91	4.53	3.40	14.72
27	0.3746	0.92	4.58	3.43	14.56
28	0.3474	0.84	4.22	3.16	15.80
29	0.3478	0.84	4.22	3.17	15.78
30	0.3276	0.79	3.96	2.97	16.85
31	0.3444	0.84	4.18	3.13	15.95
32	0.3577	0.87	4.35	3.27	15.31
33	0.3601	0.88	4.39	3.29	15.20
34	0.3392	0.82	4.11	3.08	16.22
35	0.3255	0.79	3.93	2.95	16.96
36	0.3187	0.77	3.84	2.88	17.36

Macrophage Co-culture
Figures 3E and S6D-E

BSA Standard	
mg/ml	**A562**
1.5	0.7127
1	0.4884
0.75	0.3784
0.5	0.2959
0.25	0.1573
0.125	0.0864
slope	0.45
y-intercept	0.05
R2	0.996

			dilution factor	**µl lysate**	
Sample	**A562**	**mg/ml**	**1:10**	**30 µg**	**µl buffer**
B244 1	0.2987	0.57	5.65	5.31	24.69
2	0.2576	0.47	4.73	6.34	23.66
3	0.4949	1.00	10.05	2.99	27.01
4	0.3231	0.62	6.20	4.84	25.16
5	0.2578	0.47	4.74	6.33	23.67
6	0.2938	0.55	5.54	5.41	24.59
7	0.2598	0.48	4.78	6.27	23.73
8	0.2796	0.52	5.23	5.74	24.26
9	0.2841	0.53	5.33	5.63	24.37
10	0.2743	0.51	5.11	5.87	24.13
11	0.2578	0.47	4.74	6.33	23.67
12	0.2095	0.37	3.66	8.20	21.80
13	0.2321	0.42	4.16	7.21	22.79
14	0.2436	0.44	4.42	6.79	23.21
15	0.2409	0.44	4.36	6.88	23.12
16	0.2563	0.47	4.70	6.38	23.62
17	0.293	0.55	5.53	5.43	24.57
18	0.256	0.47	4.70	6.39	23.61
19	0.2704	0.50	5.02	5.98	24.02
20	0.2569	0.47	4.72	6.36	23.64

21	0.253	0.46	4.63	6.48	23.52
22	0.2717	0.50	5.05	5.94	24.06
23	0.2298	0.41	4.11	7.30	22.70
24	0.2707	0.50	5.03	5.97	24.03
25	0.2882	0.54	5.42	5.54	24.46
26	0.2632	0.49	4.86	6.17	23.83
27	0.2354	0.42	4.24	7.08	22.92
28	0.2798	0.52	5.23	5.73	24.27
29	0.3024	0.57	5.74	5.23	24.77
30	0.3047	0.58	5.79	5.18	24.82
31	0.2373	0.43	4.28	7.01	22.99
32	0.2518	0.46	4.60	6.52	23.48
33	0.2947	0.56	5.56	5.39	24.61
34	0.2949	0.56	5.57	5.39	24.61
35	0.2309	0.41	4.14	7.25	22.75
36	0.2639	0.49	4.88	6.15	23.85
37	0.3477	0.68	6.75	4.44	25.56
38	0.3857	0.76	7.60	3.95	26.05
39	0.2552	0.47	4.68	6.41	23.59
40	0.2702	0.50	5.02	5.98	24.02
41	0.2574	0.47	4.73	6.34	23.66
42	0.2209	0.39	3.91	7.67	22.33
43	0.2679	0.50	4.96	6.04	23.96
44	0.2324	0.42	4.17	7.20	22.80
45	0.3104	0.59	5.92	5.07	24.93
46	0.2586	0.48	4.76	6.31	23.69
47	0.26	0.48	4.79	6.27	23.73
48	0.2686	0.50	4.98	6.02	23.98
49	0.4874	0.99	9.88	3.04	26.96
50	0.2878	0.54	5.41	5.54	24.46

In vivo tumor cells
Figures 3E and S6D-E

BSA Standard	
mg/ml	**A562**
2	0.8156
1.5	0.6408
1	0.4658
0.75	0.3631
0.5	0.2684
0.25	0.1536
0.125	0.0998
slope	0.38
y-intercept	0.07
R2	0.997

			dilution factor	**µl lysate**	
Sample	**A562**	**mg/ml**	**1:10**	**40 µg**	**µl buffer**
6	0.6023	1.40	14.03	2.85	27.15
7	0.5852	1.36	13.59	2.94	27.06
8	0.5296	1.21	12.13	3.30	26.70
9	0.6217	1.45	14.54	2.75	27.25
10	0.5205	1.19	11.89	3.37	26.63
11	0.5307	1.22	12.15	3.29	26.71
12	0.5474	1.26	12.59	3.18	26.82
13	0.5149	1.17	11.74	3.41	26.59
14	0.5165	1.18	11.78	3.40	26.60
15	0.5421	1.25	12.45	3.21	26.79
16	0.5471	1.26	12.59	3.18	26.82
17	0.7501	1.79	17.91	2.23	27.77
18	0.8838	2.14	21.42	1.87	28.13
19	0.4134	0.91	9.08	4.41	25.59

Tumor and Organ Labeling
Figure 4 and S7

BSA Standard	
mg/ml	**A562**
2	0.9593
1.5	0.6826
1	0.5117
0.75	0.3873
0.5	0.2956
0.25	0.1769
0.125	0.1127
slope	0.44
y-intercept	0.06
R2	0.996

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
B231 2-1 kd	0.901	1.91	19.15	14.36	3.48
1-1 KD	0.7182	1.50	14.97	11.22	4.45
1-1 LV	0.9771	2.09	20.89	15.67	3.19
1-1 SP	0.3944	0.76	7.56	5.67	8.82
1-1 Int	0.3608	0.68	6.79	5.09	9.82
1-1 PN	1.1307	2.44	24.40	18.30	2.73
1-1 HT	0.3951	0.76	7.57	5.68	8.80
1-1 LG	0.3645	0.69	6.87	5.15	9.70
1-1 BR	0.8829	1.87	18.73	14.05	3.56
1-1 Tu	0.5201	1.04	10.43	7.82	6.39
1-2 Tu	0.6886	1.43	14.29	10.72	4.67
1-3 Tu	0.7185	1.50	14.97	11.23	4.45
2-1 Tu	0.5364	1.08	10.81	8.10	6.17
2-2 Tu	0.5065	1.01	10.12	7.59	6.59
2-3 Tu	0.5741	1.17	11.67	8.75	5.71
B232 1 KD	0.6287	1.29	12.92	9.69	5.16
1 LV	1.2314	2.67	26.71	20.03	2.50
1 SP	0.3522	0.66	6.59	4.94	10.12
1 Int	0.6375	1.31	13.12	9.84	5.08
1 PN	0.4936	0.98	9.83	7.37	6.78

1 HT	0.5853	1.19	11.92	8.94	5.59
1 LG	0.4236	0.82	8.22	6.17	8.11
1 BR	0.7117	1.48	14.82	11.11	4.50
4 KD	0.6931	1.44	14.39	10.79	4.63
4 LV	0.9588	2.05	20.47	15.35	3.26
4 SP	0.3818	0.73	7.27	5.45	9.17
4 INT	0.583	1.19	11.87	8.90	5.62
4 PN	0.4986	0.99	9.94	7.46	6.71
4 HT	0.6079	1.24	12.44	9.33	5.36
4 LG	0.44	0.86	8.60	6.45	7.75
4 BR	0.6677	1.38	13.81	10.36	4.83
NP KD	0.6723	1.39	13.91	10.44	4.79
NP LV	1.1614	2.51	25.11	18.83	2.66
NP SP	0.4795	0.95	9.50	7.13	7.02
NP INT	0.4545	0.89	8.93	6.70	7.46
NP PN	0.8637	1.83	18.29	13.72	3.64
NP HT	0.4991	1.00	9.95	7.46	6.70
NP LG	0.538	1.08	10.84	8.13	6.15
NP BR	0.7038	1.46	14.64	10.98	4.56
Tu 2	0.3399	0.63	6.31	4.73	10.57
Tu 3 big	1.039	2.23	22.31	16.73	2.99
Tu 3 sm	0.7857	1.65	16.51	12.38	4.04

4T1 Lungs
Figure 5C

BSA Standard	
mg/ml	**A562**
2	0.9885
1.5	0.79063333
1	0.5095
0.75	0.39603333
0.5	0.2886
0.125	0.09216667
slope	0.48
y-intercept	0.04
R2	0.997

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
B134-23	0.7787	1.35	13.53	10.14	4.93
24	0.7252	1.40	13.96	10.47	4.78
25	0.7322	1.46	14.58	10.94	4.57
26	0.7594	1.50	15.00	11.25	4.44
27	0.7357	1.47	14.67	11.01	4.54
28	0.8311	1.52	15.22	11.41	4.38
29	0.7074	1.42	14.22	10.66	4.69
30	0.8088	1.61	16.08	12.06	4.15
31	0.7561	1.39	13.86	10.40	4.81
32	0.7372	1.45	14.47	10.86	4.61
33	0.7447	1.51	15.06	11.30	4.43
34	0.731	1.48	14.82	11.12	4.50
35	0.6953	1.35	13.51	10.13	4.93
36	0.7259	1.38	13.81	10.36	4.83
37	0.7208	1.46	14.59	10.94	4.57
38	0.9791	1.96	19.63	14.72	3.40
39	0.7219	1.39	13.94	10.46	4.78
40	0.7591	1.50	15.00	11.25	4.44
41	0.756	1.54	15.37	11.53	4.34

42	0.7173	1.42	14.24	10.68	4.68
43	0.6903	1.43	14.35	10.76	4.65
44	0.7537	1.50	14.97	11.23	4.45
45	0.7217	1.52	15.19	11.39	4.39

4T1 tumors
Figure 5D

BSA Standard	
mg/ml	**A562**
2	0.97043333
1.5	0.75653333
1	0.50486667
0.75	0.3915
0.5	0.29296667
0.25	0.15243333
0.125	0.08183333
slope	0.47
y-intercept	0.04
R2	0.999

			dilution factor	**4x buffer**	**loaded(□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
B134-1	0.6717	1.36	13.63	10.22	4.89
2	0.4929	1.06	10.59	7.94	6.30
3	0.7264	1.48	14.81	11.11	4.50
4	0.5701	1.16	11.64	8.73	5.73
5	0.6774	1.39	13.88	10.41	4.80
6	0.7102	1.46	14.62	10.96	4.56
7	0.7023	1.44	14.41	10.81	4.63
8	0.7687	1.64	16.36	12.27	4.07
9	0.7999	1.64	16.44	12.33	4.06
10	0.702	1.43	14.32	10.74	4.66
11	0.6701	1.35	13.47	10.10	4.95
12	0.6174	1.23	12.25	9.19	5.44
13	0.4649	0.90	9.05	6.79	7.37
14	0.5164	1.02	10.23	7.67	6.52
15	0.7532	1.53	15.30	11.48	4.36
16	0.7051	1.49	14.92	11.19	4.47
17	0.93	1.94	19.36	14.52	3.44
18	0.5332	1.10	10.98	8.23	6.07
19	0.5705	1.14	11.44	8.58	5.83

20	0.4964	0.99	9.86	7.39	6.76
21	0.6392	1.28	12.79	9.59	5.21
22	0.7028	1.44	14.44	10.83	4.62

caspase cross-reactivity
Figure S4

BSA Standard	
mg/ml	**A562**
2	0.9831
1.5	0.6861
1	0.5004
0.75	0.3911
0.5	0.2823
0.25	0.1712
0.125	0.0846
slope	0.46
y-intercept	0.04
R2	0.995

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**50 µg**
1	0.3588	0.69	6.92	5.19	9.64
2	0.4419	0.87	8.73	6.55	7.63
3	0.3856	0.75	7.50	5.63	8.89
4	0.4225	0.83	8.31	6.23	8.02
5	0.442	0.87	8.74	6.55	7.63
6	0.4427	0.88	8.75	6.56	7.62
7	0.4767	0.95	9.49	7.12	7.02
8	0.4328	0.85	8.53	6.40	7.81

LE28 v LP-1 in intact RAW cells: Figure S5B
LE28 dose curve in COLO205 cells: Figure S3B
LE28 time course in COLO205 cells: FigureS3C

BSA Standard	
mg/ml	**A562**
2	0.9557
1.5	0.6852
1	0.5012
0.75	0.3845
0.5	0.2772
0.125	0.0813
slope	0.45
y-intercept	0.04
R2	0.996

			dilution factor	**4x buffer**	**Loaded (□l)**
Sample	**A562**	**mg/ml**	**1:10**	**x 0.75**	**30 µg**
B239 1	0.1487	0.25	2.45	1.84	16.31
2	0.1887	0.33	3.34	2.50	11.99
3	0.1457	0.24	2.39	1.79	16.76
4	0.1999	0.36	3.58	2.69	11.16
5	0.1656	0.28	2.83	2.12	14.15
6	0.1508	0.25	2.50	1.87	16.00
7	0.1854	0.33	3.26	2.45	12.26
8	0.1255	0.19	1.94	1.46	20.61
B240 1	0.3548	0.70	7.01	5.26	5.71
2	0.4553	0.92	9.23	6.92	4.34
3	0.4088	0.82	8.20	6.15	4.88
4	0.4389	0.89	8.86	6.65	4.51
5	0.3109	0.60	6.04	4.53	6.63
6	0.3458	0.68	6.81	5.11	5.88
7	0.3975	0.80	7.95	5.96	5.03
B241 1	0.2967	0.57	5.72	4.29	6.99
2	0.288	0.55	5.53	4.15	7.23
3	0.4146	0.83	8.33	6.25	4.80
4	0.3573	0.71	7.06	5.30	5.66
5	0.3081	0.60	5.97	4.48	6.69

6	0.3069	0.59	5.95	4.46	6.72
7	0.2203	0.40	4.03	3.03	9.91

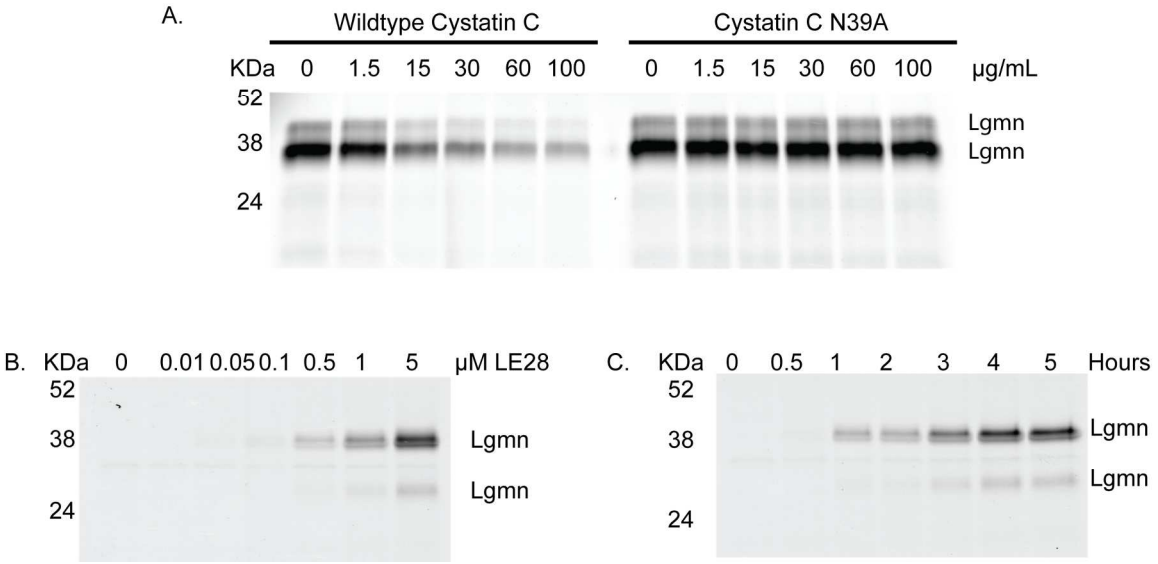


Figure S4. (A) Competition assay in which RAW cell extracts were pretreated with either recombinant Cystatin C (either WT or N39A as indicated) followed by labeling with LE28. (B) Dose dependent labeling of legumain by LE28 in intact COLO205 colorectal cancer cells. (C) Time course of LE28 labeling in intact COLO205 cells.

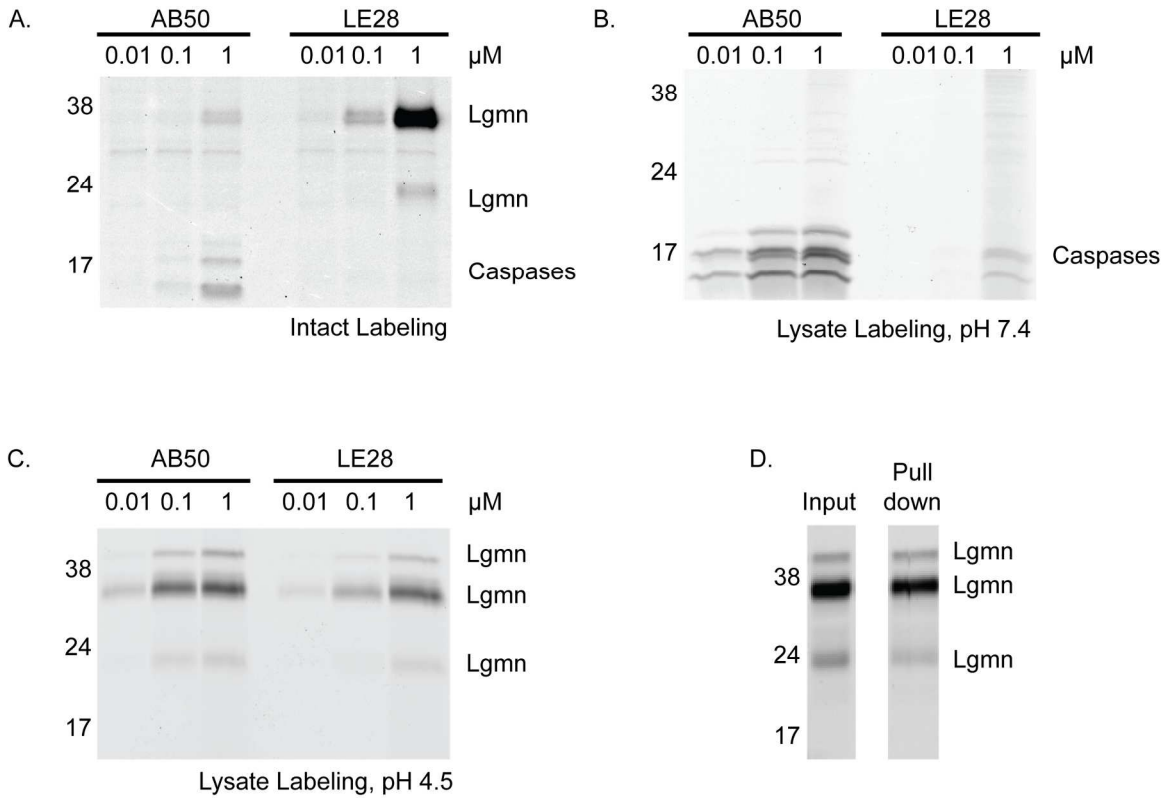


Figure S5. Assessing reactivity towards caspases. (A) Fluorescent SDS-PAGE comparing labeling of intact apoptotic COLO205 cells by AB50 or LE28. (B) Comparison of AB50 and LE28 labeling in apoptotic lysates, pH 7.4. (C) Comparison of AB50 and LE28 labeling in apoptotic lysates, pH 45. (D) Immunoprecipation from COLO205 lysates labeled with LE28 using a legumain specific antibody.

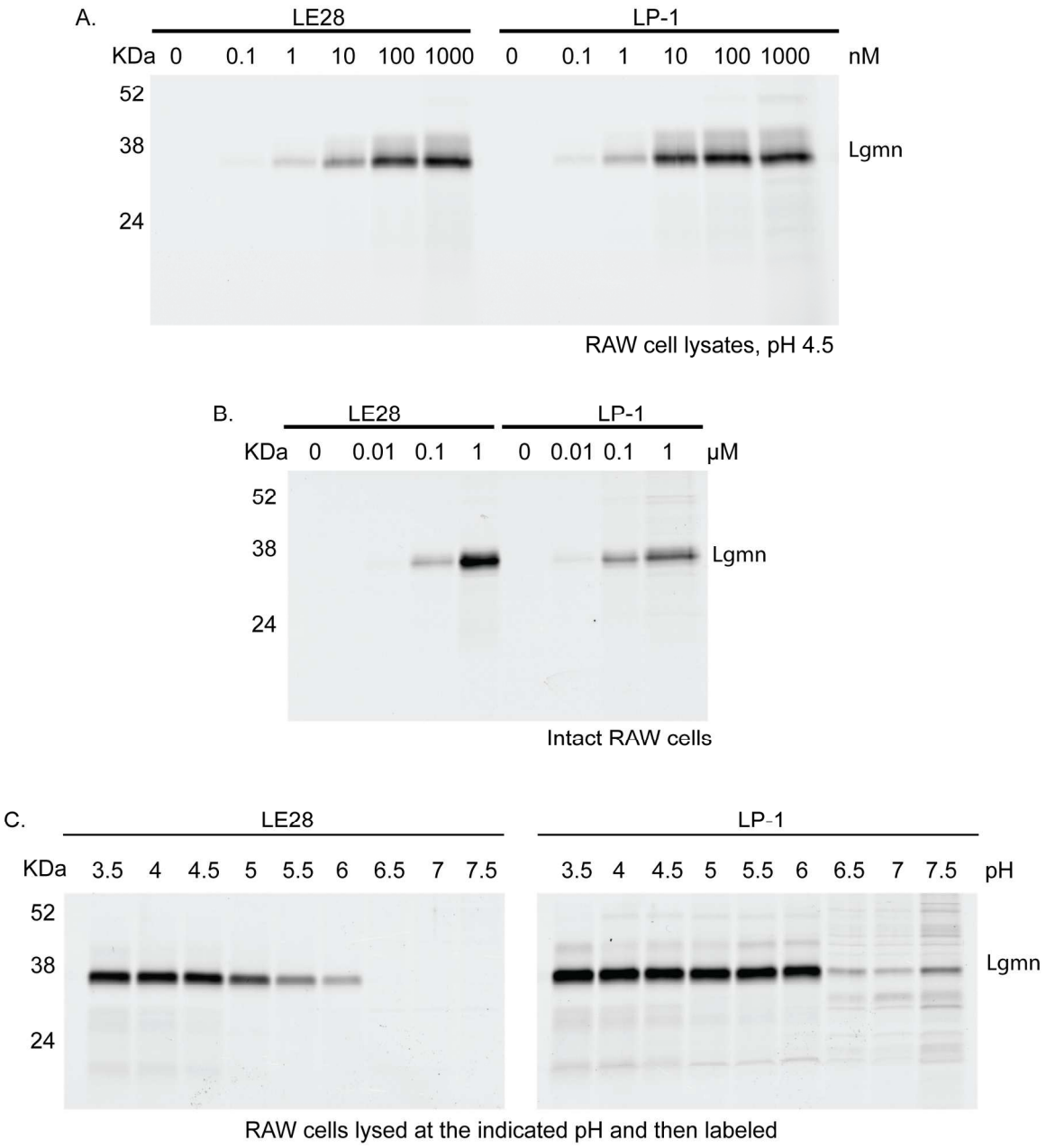


Figure S6. Direct comparison of LE28 and LP-1 labeling in RAW cells**.** (A) Fluorescent SDS-PAGE of RAW cells labeled with either LE28 or LP-1. (B) Comparison of LE28 and LP-1 labeling in intact RAW cells. (C) pH-dependence of LE28 and LP-1 labeling of legumain in RAW cell lysates.

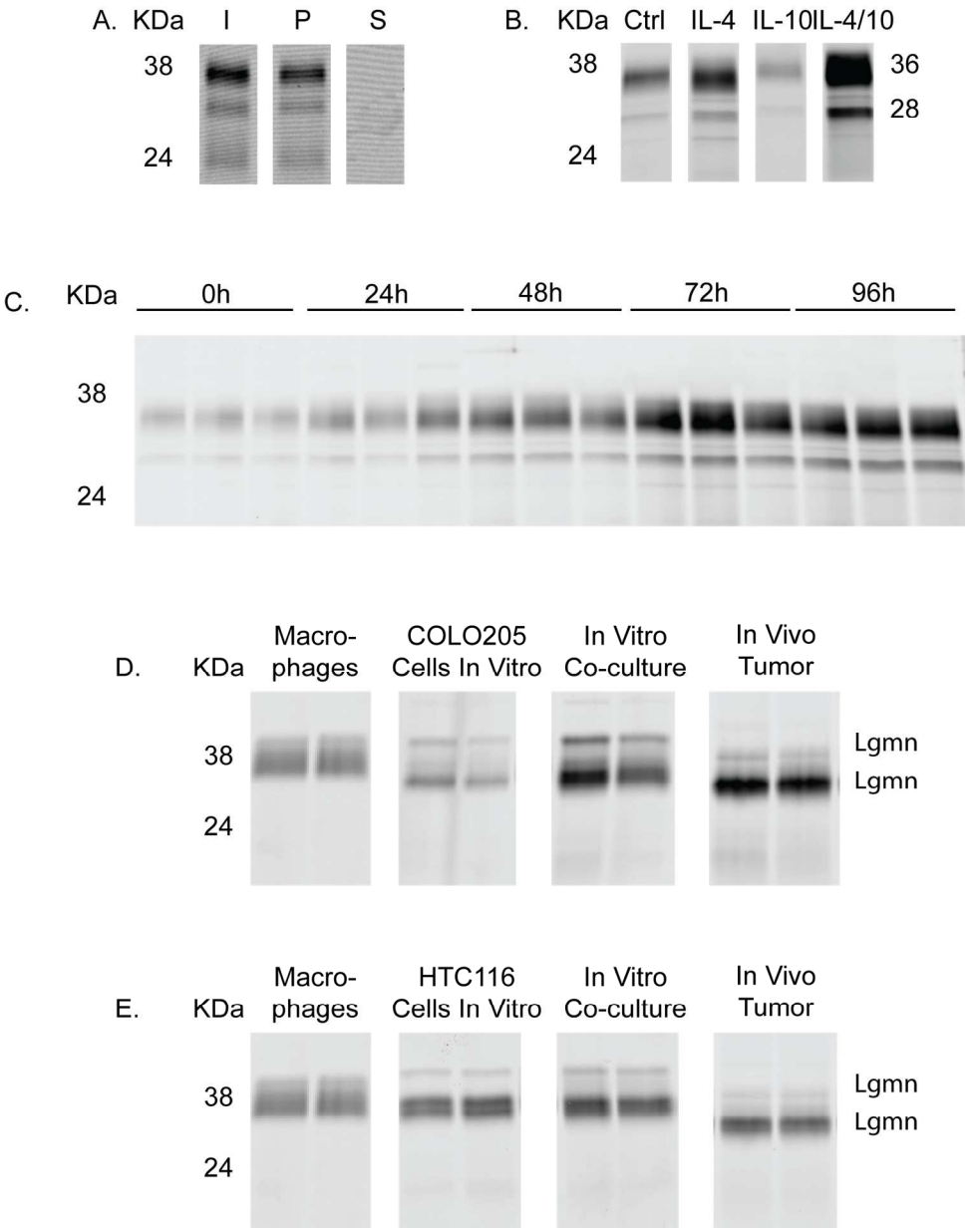


Figure S7. Regulation of legumain activity. (A) Immunoprecipitation of IL-4/-10 sample in (Figure 3a) using a legumain specific antibody to confirm the identity of the bands. I-input, P-pulldown, S-supernatant. (B) Legumain activity in primary macrophages stimulated with the indicated cytokine, followed by labeling of lysates with LE28 and subsequent fluorescent SDS-PAGE. (C) Time course of legumain activation in primary macrophages that were exposed to IL4/10. Cells were lysed and then labeled with LE28 followed by SDS-PAGE (D) Legumain activation in macrophages alone, COLO205 tumor cells, or in 1:1 co-culture. The rightmost column shows activity in tumor cells that were implanted subcutaneously in nude mice for one week. For each group, lysates were prepared and labeled with LE28. (E) Identical experiment as in (D) except the tumor cells were HCT-166 colon cancer cells.

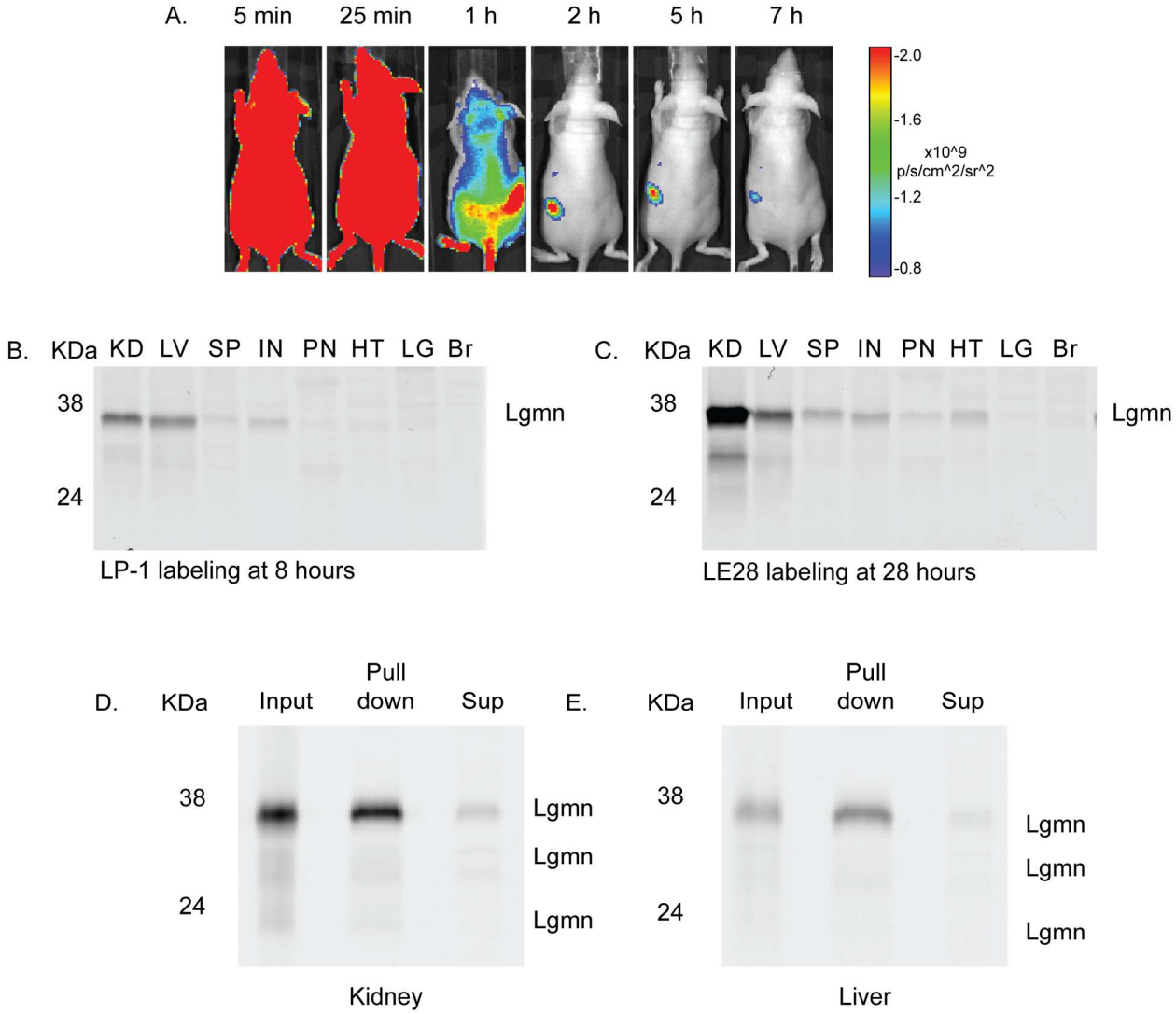


Figure S8. (A) Normal mice were injected with LP-1 and then imaged for fluorescence by IVIS over time. (B) Tissues were removed from mice in (A) and analyzed for LP-1 labeling by fluorescent SDS-PAGE. KD = kidney, LV = liver, SP = spleen, IN = intestine, PN = pancreas, HT = heart, LG = lung, and BR = brain. (C) Fluorescent SDS-PAGE of tissues harvested 28 hours after LE28 injection. (D-E) Immunoprecipitation of kidney and liver lysates from LE28-injected mice using a legumain-specific polyclonal antibody.

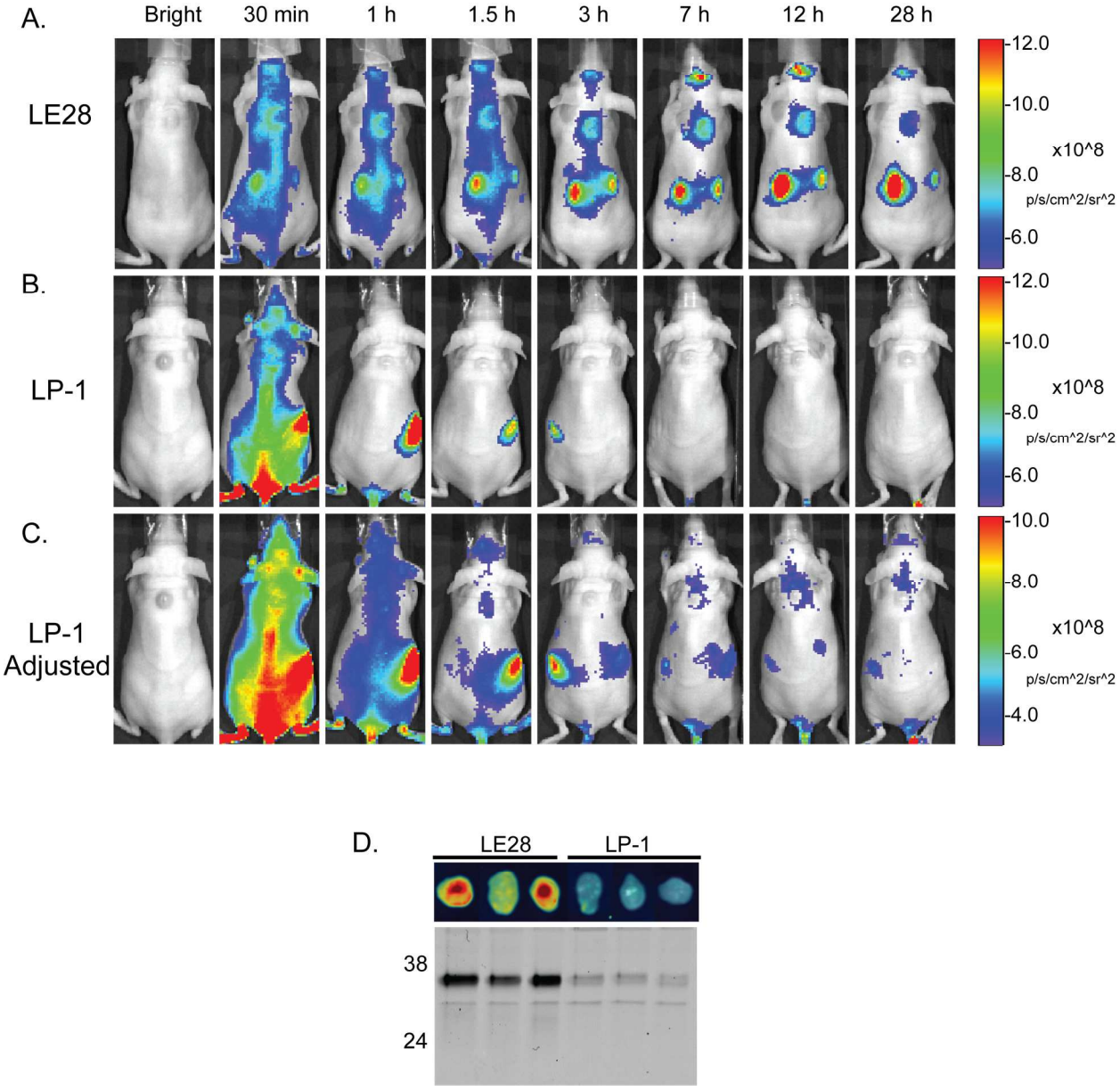


Figure S9. (A-B) Mice bearing HCT-116 human colon cancer xenografts were injected with either LE28 (A) or LP-1 (B) and then imaged using the IVIS system at various timepoints over the course of 28 hours. As indicated by the scale bars, the mice in A and B are shown with the same gain settings to directly compare the brightness of LE28 and LP-1. In (C), the same mice presented in (B) are shown at an increased gain setting to show the most optimal constrast for LP-1. (D) Ex vivo imaging of tumors excised at the end of the time course and subsequent SDS-PAGE analysis on lysates prepared from the same tumors. Note: the data for LE28 is presented in Figure 4 but is reiterated here for ease of comparison.