SI Appendix for

Mapping the Binding Interface of ERK and Transcriptional Repressor Capicua Using Photocrosslinking

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SI Materials and Methods

Plasmid Construction

The plasmid encoding biotinylated ERK was constructed by standard PCR amplification of an N-terminal 18 amino acid biotin acceptor peptide (BAP) sequence and the rat ERK2 sequence containing a C-terminal His_6 tag and fusion of the two sequences by overlap PCR. The gene encoding BAP-ERK2 was digested and ligated into pASK75 (1). To construct a plasmid for non-biotinylated, N-terminally His_6 tagged ERK (rat ERK2 and *Drosophila* ERK, Rolled), the ERK gene was amplified by PCR, digested, and ligated into pQE80. Biotinylated ERK was used for fly Cic crosslinking and Cic variant screens and non-biotinylated ERK was used for human cic crosslinking and ERK variant screens. GST-fused human and *Drosophila* Cic fragment plasmids were made by PCR amplification of C-terminally His_6 tagged Cic fragments, digestion, and ligation into pGEX4t-1. Plasmids containing mutants of *Drosophila*/human Cic fragments or rat ERK2 were constructed by overlap PCR of 5'- and 3'-ends of the genes produced by standard PCR using primers containing the desired mutation, digestion, and ligation into pQE80 (ERK) or pGEX4t-1 (Cic fragments).

Protein Expression and Purification

Plasmids containing non-biotinylated ERK variants were transformed into *E. coli* BL21 competent cells. Overnight cultures were sub-cultured into LB medium supplemented with ampicillin (100 μg/mL) to a starting OD_{600} of 0.02, and cultures were grown at 37 °C until they reached OD_{600} 1.0. Protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cultures were moved to room temperature and allowed to grow for 4-6 hours. Bacterial cell pellets were harvested by centrifugation and stored at -20 °C. GSTCic and GSThCic variants were expressed in the same manner as the non-biotinylated ERK variants with the exception of growth temperature, which was maintained at 37 °C for the duration of the expression.

For the expression of biotinylated ERK, the plasmid harboring BAP-ERK2 and a plasmid encoding the biotin ligase BirA (expressed constitutively) were co-transformed into *E. coli* BL21 competent cells. LB medium supplemented with ampicillin and kanamycin (100 μg/mL and 35 μg/mL, respectively) was inoculated with overnight culture to a starting OD_{600} of 0.02. Cultures were grown at 37 °C, and at OD_{600} of 0.2 2 mg/L D-biotin was added to the culture. At OD_{600} 0.8-1.0, protein expression was induced with the addition of anhydrotetracycline to a final concentration of 0.2 μg/mL. Cultures were moved to room temperature and allowed to grow for 4-6 hours. Bacterial cell pellets were harvested by centrifugation and stored at -20 °C.

For the expression of AzF-incorporated GSTCic and ERK variants, plasmids harboring the protein of interest and the AzF-RS/mutRNA$_{CUA}$ pair (2) on a pEVOL plasmid (3) were co-transformed into *E. coli* BL21 competent cells. LB medium supplemented with ampicillin and chloramphenicol (100 μg/mL and 50 μg/mL, respectively) was inoculated with overnight culture to a starting OD_{600} of 0.02 and the cultures were grown to an OD_{600} of 1.0. AzF was added to the culture to a final concentration of 3 mM and protein expression was induced with 1 mM IPTG for the protein of interest and 2% arabinose for the aminoacyl-tRNA synthetase. Cultures were grown at 37 °C (GSTCic) or 25 °C (ERK) for 4-6 hours. Bacterial cell pellets were harvested by centrifugation and stored at -20 °C.

For all protein purifications, cell pellets were resuspended in 10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8 and lysed by treatment with lysozyme and sonication on ice. Cell debris was removed by centrifugation and the supernatant was sterile filtered. All proteins were purified from clarified lysate using Ni-NTA agarose resin (Qiagen) following the manufacturer's recommendations. GST-fused proteins were further purified using Glutathione Sepharose resin

(GE Healthcare) following the manufacturer's recommendations. After purification, proteins were buffer exchanged into PBS containing 10% glycerol using PD-10 desalting columns (Bio-Rad). If proteins were to be stored for more than one week, 30 µL aliquots were prepared, snap frozen in liquid nitrogen, and stored at -80 °C.

GST Pull-Down

For the GSTCic alanine screen, human Cic N-terminal fragment screen, and GSThCic fragment screen experiments (testing binding of different Cic fragments/variants to WT ERK), the GST-containing bait protein was immobilized on Glutathione Sepharose resin directly from bacterial lysates. Cultures (10 mL of LB medium) supplemented with ampicillin (100 µg/mL) were inoculated with overnight cultures harboring plasmids encoding a GST-fused Cic fragment to a starting OD_{600} of 0.02. Cultures were grown at 37 °C to OD_{600} 0.6-0.8 and induced with 1 mM IPTG. Cultures were grown for an additional 2 hours at 37 °C and bacterial cell pellets were harvested by centrifugation. Pellets were resuspended in 1 mL PBS and lysed by treatment with lysozyme and sonication on ice. Twenty-five µL Glutathione Sepharose resin was added to the lysate and incubated at 4 °C for 1 hour rotating end-over-end. The resin was settled by centrifugation and the supernatant discarded. The resin was washed twice with 1 mL PBS to obtain a pure GST-fused Cic fragment immobilized on Glutathione Sepharose resin. For the ERK variant screen (testing binding of different ERK docking domain variants to WT GSTCic or GSThCic), the GST-containing bait protein was immobilized on Glutathione Sepharose resin from purified protein stocks (preparation of purified protein described above). Twenty-five µL Glutathione Sepharose resin was washed with 500 µL PBS and incubated on ice for 30 min with 0.15 nmol protein in PBS to obtain GST-fused Cic fragment immobilized on the resin. For all experiments, purified ERK was added to the immobilized Cic fragment at a final concentration of 1 µM and final volume of 75 µL and incubated on ice for 30 min. The resin was allowed to settle and the supernatant was discarded. The resin was washed twice with 1 mL PBS to remove any remaining unbound ERK. Thirty µL 10 mM reduced Glutathione, 50 mM Tris, pH 8.0 was added and incubated on ice for 30 min to elute the GST-fused Cic fragment and bound ERK. The supernatant was removed from the settled resin, mixed with 2x SDS sample buffer, and analyzed by protein gel and Western blot for the presence of Cic and ERK.

Biolayer Interferometry

Binding by BLI was performed on the ForteBio BLItz system. All samples were prepared prior to a binding experiment in PBS at a concentration 3x above the target concentration and stored on ice until use. Just prior to a trial, the ligand and analyte were diluted 3-fold in room temperature PBS containing 0.2% BSA and 0.02% Tween20. For ERK-GSTCic binding experiments, biotinylated ERK at a concentration of 0.5 µM was loaded onto a SA coated biosensor for 120 seconds and the biosensor was washed in buffer for 60 seconds. GSTCic (concentration 50-800 nM) was introduced and association was measured for 90 seconds. The biosensor was moved to buffer and dissociation was measured for 390 seconds. For GSThCic-ERK binding experiments, GSThCic at a concentration of 5µM was loaded onto an anti-GST antibody coated biosensor for 120 seconds and washed in buffer for 30 seconds. ERK (concentration 100-1600 nM) was introduced and association was measured for 20 seconds. The biosensor was moved to buffer and dissociation was measured for 60 seconds. For both experiments, the K_D was calculated by global fit to five different analyte concentration trials using ForteBio BLItz Pro software; the reported K_Ds are an average of 3 such fits and the error represents one standard deviation from the mean.

UV Photocrosslinking

Prior to crosslinking, ERK (*Drosophila* or human, 1 μM) and GSTCic or GSThCic (2 μM) in PBS were incubated at 4 °C for 30 min in 200 uL in a single well of a 96-well polystyrene plate. The plate was covered with a polystyrene lid and moved onto the surface of a UV transilluminator (UVP, model LMW-20) and irradiated with 365 nm light at 4 °C. The maximum observed crosslinking efficiency was obtained by irradiating for 1 hour in 15 min increments allowing 10 min for cooling in between irradiation periods to avoid overheating. After irradiation, the sample was removed from the 96-well plate, mixed with 2x SDS sample buffer, and analyzed by protein gel and Western blot for the presence of a slow-migrating, crosslinked heterodimer. Crosslinking efficiency was estimated by measuring the band intensity of ERK, Cic, and the crosslinked adduct from a protein gel of the photoreaction product using ImageJ. Crosslinking efficiency is defined as the intensity of the crosslinked adduct band divided by the total intensity of the ERK, Cic, and adduct bands.

Mass Spectrometry

The Coomassie stained gel bands containing photocrosslinked proteins were subjected to in-gel digestion. Briefly, gel bands were diced into 1 mm cubes, washed, subjected to thiol reduction of Cystein by Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and alkylation with iodoacetamide. Trypsin digestion was performed overnight at 37 °C using Trypsin Gold (Promega). Peptides were extracted from the gel pieces, concentrated in SpeedVac, and desalted using a STAGE tip.

LC-MS/MS on desalted peptides from the *Drosophila* ERK-Cic and mammalian ERK-hCic adduct was performed using an Easy nLC 1000 Ultra nano-flow capillary ultra-high performance liquid chromatography (UPLC) system (Thermo Fisher Scientific) connected to an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific). A Flex ion source (Proxeon) was used as the nano spray ionization source. A capillary trap column (100 μm I.D. x 40 mm, packed with 100 A' 3 μm Magic AQ3 C18 resin, Michrom) was used to concentrate and load the sample at a flow rate of 5 uL/min for 5 min. Peptides were separated on an analytical column (75 μm I.D. x 450 mm, packed with 100 A' 3 μm Magic AQ3 C18 resin, Michrom) with a flow rate of 300 nL/min (*Drosophila* ERK-Cic experiment) or 250 nL/min (mammalian ERK-hCic experiment). Both trap column and analytical column were heated to 50 °C in a column heater. A linear gradient of 5% B to 35% B (buffer A, 0.1% formic acid in water; buffer B, 0.1% formic acid in acetonitrile) in 160 minutes (*Drosophila* ERK-Cic experiment) or 95 minutes (mammalian ERK-hCic experiment) was utilized to separate peptides. Nanospray ionization was performed on an Orbitrap Elite mass spectrometer using 1.8 kV source voltage and capillary temperature of 275 °C (*Drosophila* ERK-Cic experiment) or 250 °C (mammalian ERK-hCic experiment). Full scan spectra, in positive mode, were acquired in Orbitrap over m/z range of 335-1800 with 120,000 resolution power and MS/MS fragmentation spectra were acquired in linear LTQ ion trap using collision induced dissociation (CID) for the top 20 (*Drosophila* ERK-Cic experiment) or 15 (mammalian ERK-hCic experiment) most abundant peptides with a minimum signal intensity of 2000. Poly dimethyl siloxane, (C12H36O6Si6)H+, m/z = 445.12002 was used as lock mass. Injection waveforms function was activated. Dynamic exclusion was set at 120 seconds.

The mammalian ERK-Cic cross linked sample was run on a Nano Ultra 2D Plus capillary UPLC system (AB Sciex) interfaced with a LTQ-Orbitrap XL hybrid mass spectrometer. A nanospray source made in-house was used as the ionization interface. The same trap and analytical column settings, buffer systems, and flow rates were used with exception of the linear gradient run time for peptide separation (35 min). The capillary temperature was 250 °C and source voltage was 1.8 kV. Full scan spectra were acquired in Orbitrap over a m/z range of 340-1800 in positive mode with a resolution power of 100,000. MS/MS spectra of the top seven most

abundant peptides with a minimum signal intensity of 2000 were acquired in linear LTQ ion trap using collision induced dissociation (CID). No lock mass was used. Injection waveform was activated and dynamic exclusion was set at 120 seconds.

References

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2. Chin JW*, et al.* (2002) Addition of p-Azido-l-phenylalanine to the Genetic Code of Escherichia coli. *J. Am. Chem. Soc.* 124(31):9026-9027.
3. Young TS, Ahmad I, Yin JA, & Schultz PG (2010) An Enhanced System for Unnatural Amino Acid Mutagenesis in E. coli. *J. Mol. Biol.* 395(2):361-374.

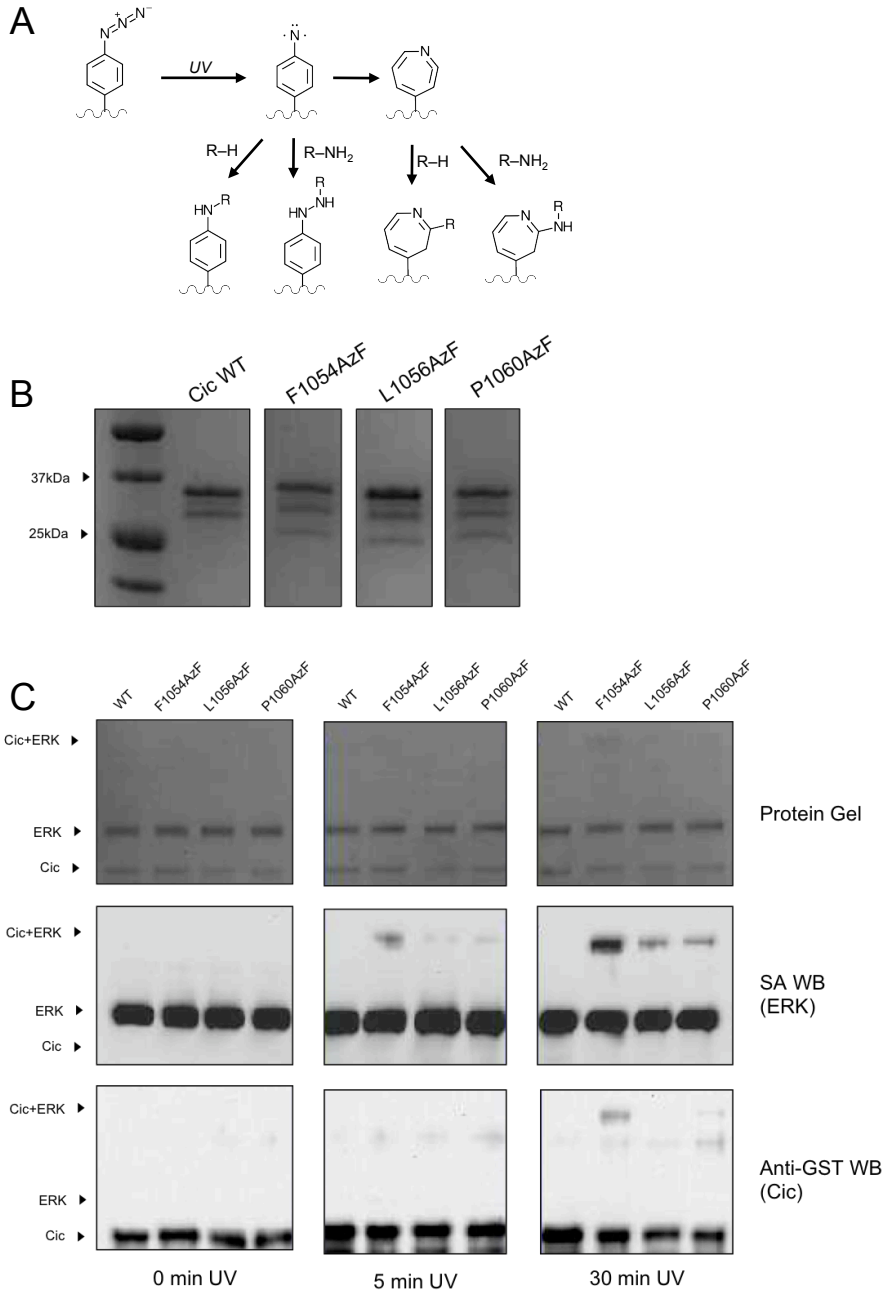

Fig. S1. Photocrosslinking of ERK and AzF-incorporated *Drosophila* Cic. *(A)* Upon irradiation by UV light, the aryl azide moeity of AzF is activated to form a reactive nitrene. The nitrene can undergo insertion into nearby C-H or N-H bonds either directly or following ring expansion through a dehydroazepine intermediate. *(B)* Incorporation of AzF into GSTCic. AzF was inserted into three positions in GSTCic (F1054, L1056, and P1060) by stop codon suppression. Expression in *E. coli* and purification by metal affinity chromatography yielded similar amounts of WT and AzF-incorporated GSTCic. *(C)* UV exposure-dependent crosslinking of ERK and AzF-containing GSTCic. WT and AzF-incorporated Cic variants were incubated with ERK and exposed to UV light for 0, 5, and 30 min. The appearance of a slow migrating species in protein gels, anti-GST Western blots (detects Cic), and streptavidin (SA) Western blots (detects biotinylated ERK) with increasing time of UV irradiation in reactions containing AzF-incorporated GSTCic indicates succesful ERK-GSTCic photocrosslinking.

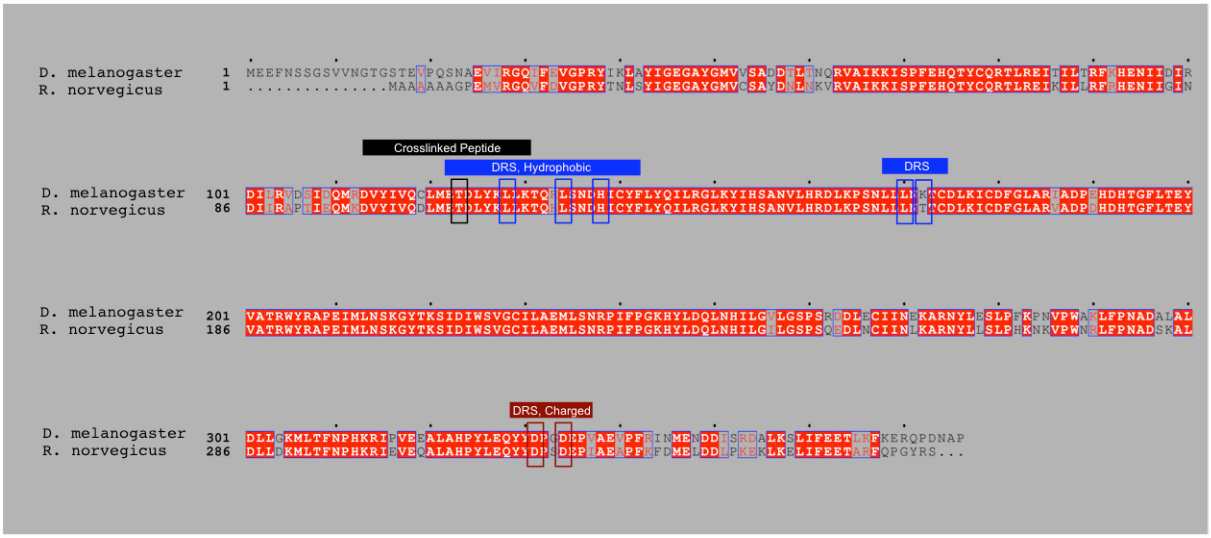

Fig. S2. Mammalian and *Drosophila* ERK are highly conserved. A sequence alignment of rat ERK2 and the *Drosophila* homolog Rolled illustrates the high level of sequence similarity (92.3%), especially in regions of the enzyme used to interact with its binding partners. The same region of *Drosophila* and rat ERK were found to crosslink to an AzF-incorporated variant of a fragment of fly Cic (shown in black). This crosslinking site is adjacent to the ERK DRS (hydrophobic and charged regions of DRS shown in blue and red, respectively). These sites are all highly conserved between organisms.

ERK (fragments) +
Cic (full-length)

	b+	2	3	4	5	6		y+	2	3	4	5	6	
1							**D**	4068.1	2034.6	1356.7	1017.8	814.4	678.9	**18**
2	215.1	108.1	72.4	54.5	43.8	36.7	**V**	3953.1	1977.1	1318.4	989.0	791.4	659.7	**17**
3	**378.2**	189.6	126.7	95.3	76.4	63.9	**Y**	3854.0	1927.5	**1285.3**	964.3	771.6	643.2	**16**
4	**491.3**	246.1	164.4	123.6	99.1	82.7	**I**	3691.0	1846.0	**1231.0**	923.5	739.0	616.0	**15**
5	590.3	295.7	197.4	148.3	118.9	99.2	**V**	3577.9	1789.4	**1193.3**	895.2	716.4	597.2	**14**
6	**718.4**	359.7	240.1	180.4	144.5	120.6	**Q**	3478.8	1739.9	**1160.3**	870.5	696.6	580.6	**13**
7	833.4	417.2	278.5	209.1	167.5	139.7	**D**	3350.8	1675.9	**1117.6**	838.4	671.0	559.3	**12**
8	946.5	473.7	316.2	237.4	190.1	158.6	**L**	3235.7	1618.4	1079.2	809.7	648.0	540.1	**11**
9	1077.5	539.3	**359.8**	270.1	216.3	180.4	**M**	3122.6	1561.8	**1041.6**	781.4	625.3	521.3	**10**
10	1206.6	603.8	402.9	302.4	242.1	201.9	**E**	2991.6	1496.3	997.9	748.7	599.1	499.4	**9**
11	3176.6	**1588.8**	1059.5	794.9	636.1	530.3	**T-Cic**	2862.6	1431.8	954.9	716.4	573.3	477.9	**8**
12	3291.6	1646.3	1097.9	823.7	659.1	549.4	**D**	**892.6**	446.8	298.2	223.9	179.3	149.6	**7**
13	3404.7	1702.8	1135.6	851.9	681.7	568.3	**L**	777.5	389.3	259.8	195.1	156.3	130.4	**6**
14	3567.8	1784.4	1189.9	**892.7**	714.4	595.5	**Y**	664.4	332.7	222.2	166.9	133.7	111.6	**5**
15	3695.8	1848.4	1232.6	924.7	740.0	616.8	**K**	501.4	251.2	167.8	126.1	101.1	84.4	**4**
16	3808.9	1905.0	**1270.3**	953.0	762.6	635.7	**L**	373.3	187.1	125.1	94.1	75.5	63.1	**3**
17	3922.0	1961.5	1308.0	981.3	785.2	654.5	**L**	260.2	130.6	87.4	65.8	52.8	44.2	**2**
18	4068.1	2034.6	1356.7	1017.8	814.4	678.9	**K**	147.1	74.1	49.7	37.5	30.2	25.4	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S1. Expected m/z of *b*- and *y*-series fragments of ERK peptide with full-length *Drosophila* Cic peptide adduct.

Cic (fragments) +
ERK (full-length)

	b+	2	3	4	5	6		y+	2	3	4	5	6	
1							**G**	4068.1	2034.6	1356.7	1017.8	814.4	678.9	**18**
2	145.1	73.0	49.0	37.0	29.8	25.0	**S**	4011.1	2006.1	1337.7	1003.5	803.0	669.4	**17**
3	260.1	130.5	87.4	65.8	52.8	44.2	**D**	3924.1	1962.5	1308.7	**981.8**	785.6	654.9	**16**
4	317.1	159.1	106.4	80.0	64.2	53.7	**G**	3809.0	1905.0	**1270.4**	**953.0**	762.6	635.7	**15**
5	445.2	223.1	149.1	112.0	89.8	75.0	**Q**	3752.0	1876.5	1251.3	938.8	751.2	626.2	**14**
6	573.2	287.1	191.7	144.1	115.5	96.4	**Q**	3624.0	1812.5	1208.7	906.7	725.6	604.8	**13**
7	2932.4	1466.7	978.2	733.9	587.3	489.6	**Z-ERK**	3495.9	1748.5	1166.0	874.7	700.0	583.5	**12**
8	3045.5	1523.3	**1015.8**	762.1	609.9	508.4	**I**	1136.7	568.8	379.6	284.9	228.1	190.3	**11**
9	3158.6	1579.8	**1053.5**	790.4	632.5	527.3	**L**	1023.6	512.3	341.9	256.7	205.5	171.4	**10**
10	3229.7	**1615.3**	1077.2	808.2	646.7	539.1	**A**	**910.5**	455.8	304.2	228.4	182.9	152.6	**9**
11	3326.7	1663.9	1109.6	832.4	666.1	555.3	**P**	**839.5**	420.2	280.5	210.6	168.7	140.8	**8**
12	3427.8	1714.4	1143.3	857.7	686.4	572.1	**T**	742.4	371.7	248.1	186.4	149.3	124.6	**7**
13	3524.8	1762.9	**1175.6**	882.0	**705.8**	588.3	**P**	**641.4**	321.2	214.5	161.1	129.1	107.7	**6**
14	3595.8	1798.4	1199.3	899.7	720.0	600.1	**A**	544.3	272.7	182.1	136.8	109.7	91.6	**5**
15	3723.9	1862.5	1242.0	931.7	745.6	621.5	**Q**	473.3	237.1	158.4	119.1	95.5	79.7	**4**
16	3837.0	1919.0	**1279.7**	960.0	768.2	640.3	**L**	345.2	173.1	115.7	87.1	69.9	58.4	**3**
17	3894.0	1947.5	1298.7	974.3	779.6	649.8	**G**	232.1	116.6	78.1	58.8	47.2	39.5	**2**
18	4068.1	2034.6	1356.7	1017.8	814.4	678.9	**R**	175.1	88.1	59.0	44.5	35.8	30.0	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S2. Expected m/z of *b*- and *y*-series fragments of *Drosophila* Cic peptide with full-length ERK peptide adduct.

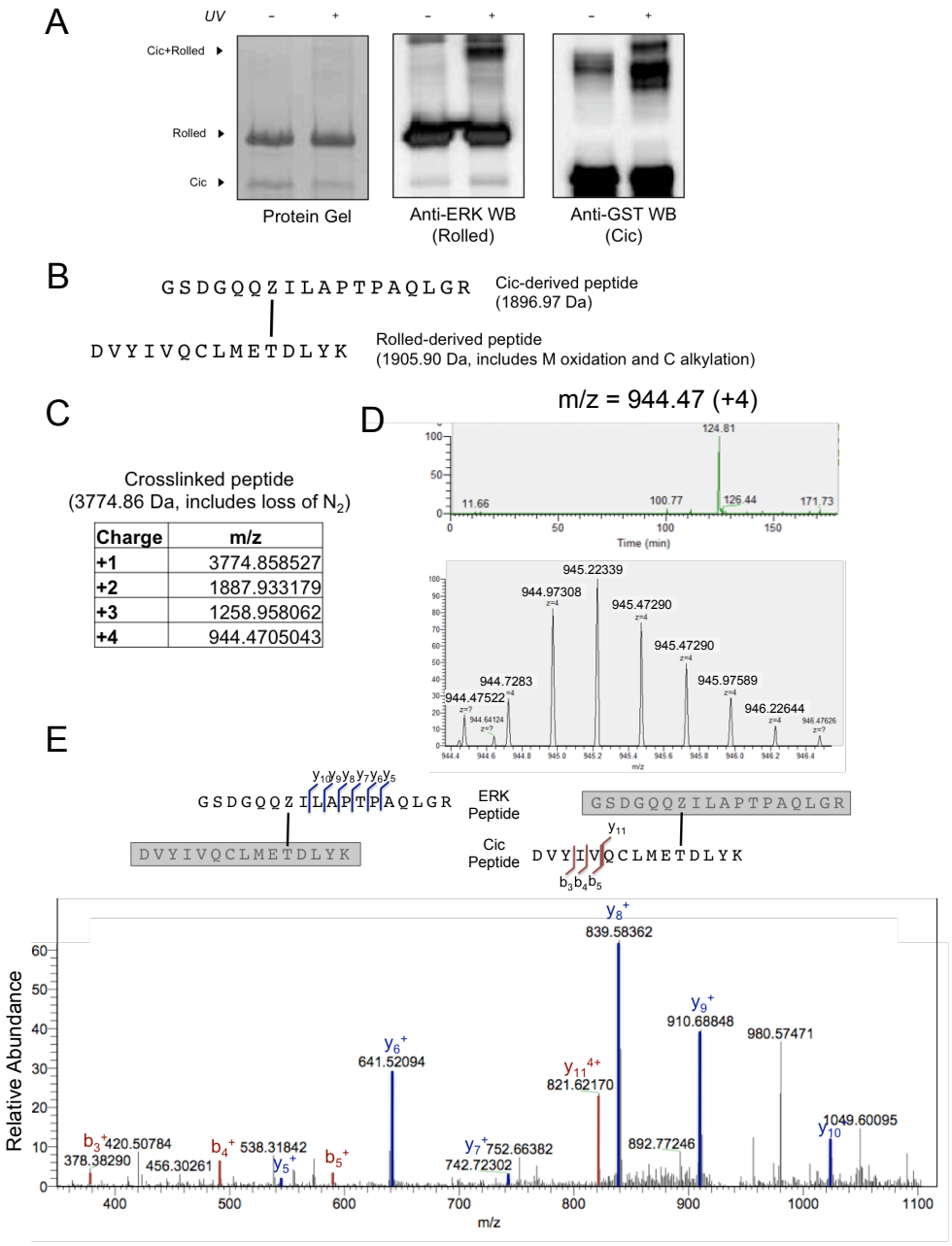

Fig. S3. Photocrosslinking of Rolled and *Drosophila* Cic and identification of crosslinked peptide by mass spectrometry. *(A)* GSTCic WT and F1054AzF were incubated with Rolled and exposed to UV light. The formation of a slow-migrating, crosslinked Rolled-Cic heterodimer was determined by protein gel, anti-ERK Western blot (detects Rolled), and anti-GST Western blot (detects GSTCic). *(B)* Sequence and mass of Cic- and ERK-derived peptides that make up the crosslinked peptide. *(C)* Expected m/z of different charge states of the Rolled-Cic crosslinked peptide. *(D)* Extracted ion chromatogram (top) and mass spectrum (bottom) of +4 charge state of the Rolled-Cic crosslinked peptide. *(E)* Tandem mass spectrometry of the crosslinked Rolled-Cic peptide. *y*-series fragments of the Cic peptide with a full-length Rolled peptide adduct are shown in blue and *b*- and *y*- series fragments of the Rolled peptide with a full-length Cic peptide adduct are shown in red.

Rolled (C alkylated, M oxidized) (fragments) +
Cic (full-length)

	b+	2	3	4	5	6		y+	2	3	4	5	6	
1							**D**	3774.9	1887.9	1259.0	944.5	755.8	630.0	**15**
2	215.1	108.1	72.4	54.5	43.8	36.7	**V**	3659.8	1830.4	1220.6	915.7	732.8	610.8	**14**
3	**378.2**	189.6	126.7	95.3	76.4	63.9	**Y**	3560.8	1780.9	1187.6	890.9	713.0	594.3	**13**
4	**491.3**	246.1	164.4	123.6	99.1	82.7	**I**	3397.7	1699.4	1133.2	850.2	680.3	567.1	**12**
5	**590.3**	295.7	197.4	148.3	118.9	99.2	**V**	3284.6	1642.8	1095.5	**821.9**	657.7	548.3	**11**
6	718.4	359.7	240.1	180.4	144.5	120.6	**Q**	3185.5	1593.3	1062.5	797.1	637.9	531.8	**10**
7	878.4	439.7	293.5	220.4	176.5	147.2	**C(alkyl)**	3057.5	1529.2	1019.8	765.1	612.3	510.4	**9**
8	991.5	496.2	331.2	248.6	199.1	166.1	**L**	2897.5	1449.2	966.5	725.1	580.3	483.7	**8**
9	1138.5	569.8	380.2	285.4	228.5	190.6	**M(ox)**	2784.4	1392.7	928.8	696.8	557.7	464.9	**7**
10	1267.6	634.3	423.2	317.6	254.3	212.1	**E**	2637.3	1319.2	879.8	660.1	528.3	440.4	**6**
11	3237.6	1619.3	1079.9	810.2	648.3	540.4	**T-Cic**	2508.3	1254.7	836.8	627.8	502.5	418.9	**5**
12	3352.6	1676.8	1118.2	838.9	671.3	559.6	**D**	538.3	269.6	180.1	135.3	108.5	90.6	**4**
13	3465.7	1733.3	1155.9	867.2	693.9	578.5	**L**	423.3	212.1	141.8	106.6	85.5	71.4	**3**
14	3628.8	1814.9	1210.3	907.9	726.6	605.6	**Y**	310.2	155.6	104.1	78.3	62.8	52.5	**2**
15	3774.9	1887.9	1259.0	944.5	755.8	630.0	**K**	147.1	74.1	49.7	37.5	30.2	25.4	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S3. Expected m/z of *b*- and *y*-series fragments of Rolled peptide with full-length *Drosophila* Cic peptide adduct.

Cic (fragments) +
Rolled (C alkylated, M oxidized) (fuil-length)

	b+	2	3	4	5	6		y+	2	3	4	5	6	
1							**G**	3774.9	1887.9	1259.0	944.5	755.8	630.0	**18**
2	145.1	73.0	49.0	37.0	29.8	25.0	**S**	3717.8	1859.4	1240.0	930.2	744.4	620.5	**17**
3	260.1	130.5	87.4	65.8	52.8	44.2	**D**	3630.8	1815.9	1210.9	908.5	727.0	606.0	**16**
4	317.1	159.1	106.4	80.0	64.2	53.7	**G**	3515.8	1758.4	1172.6	879.7	704.0	586.8	**15**
5	445.2	223.1	149.1	112.0	89.8	75.0	**Q**	3458.8	1729.9	1153.6	865.4	692.6	577.3	**14**
6	573.2	287.1	191.7	144.1	115.5	96.4	**Q**	3330.7	1665.9	1110.9	833.4	666.9	556.0	**13**
7	2639.2	1320.1	880.4	660.6	528.6	440.7	**Z-Rolled**	3202.6	1601.8	1068.2	801.4	641.3	534.6	**12**
8	2752.3	1376.6	918.1	688.8	551.3	459.6	**I**	1136.7	568.8	379.6	284.9	228.1	190.3	**11**
9	2865.4	1433.2	955.8	717.1	573.9	478.4	**L**	**1023.6**	512.3	341.9	256.7	205.5	171.4	**10**
10	2936.4	1468.7	979.5	734.9	588.1	490.2	**A**	**910.5**	455.8	304.2	228.4	182.9	152.6	**9**
11	3033.4	1517.2	1011.8	759.1	607.5	506.4	**P**	**839.5**	420.2	280.5	210.6	168.7	140.8	**8**
12	3134.5	1567.8	1045.5	784.4	627.7	523.3	**T**	**742.4**	371.7	248.1	186.4	149.3	124.6	**7**
13	3231.5	1616.3	1077.9	808.6	647.1	539.4	**P**	**641.4**	321.2	214.5	161.1	129.1	107.7	**6**
14	3302.6	1651.8	1101.5	826.4	661.3	551.3	**A**	**544.3**	272.7	182.1	136.8	109.7	91.6	**5**
15	3430.6	1715.8	1144.2	858.4	686.9	572.6	**Q**	473.3	237.1	158.4	119.1	95.5	79.7	**4**
16	3543.7	1772.4	1181.9	886.7	709.6	591.5	**L**	345.2	173.1	115.7	87.1	69.9	58.4	**3**
17	3600.7	1800.9	1200.9	900.9	721.0	601.0	**G**	232.1	116.6	78.1	58.8	47.2	39.5	**2**
18	3774.9	1887.9	1259.0	944.5	755.8	630.0	**R**	175.1	88.1	59.0	44.5	35.8	30.0	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S4. Expected m/z of *b*- and *y*-series fragments of *Drosophila* Cic peptide with full-length Rolled peptide adduct.

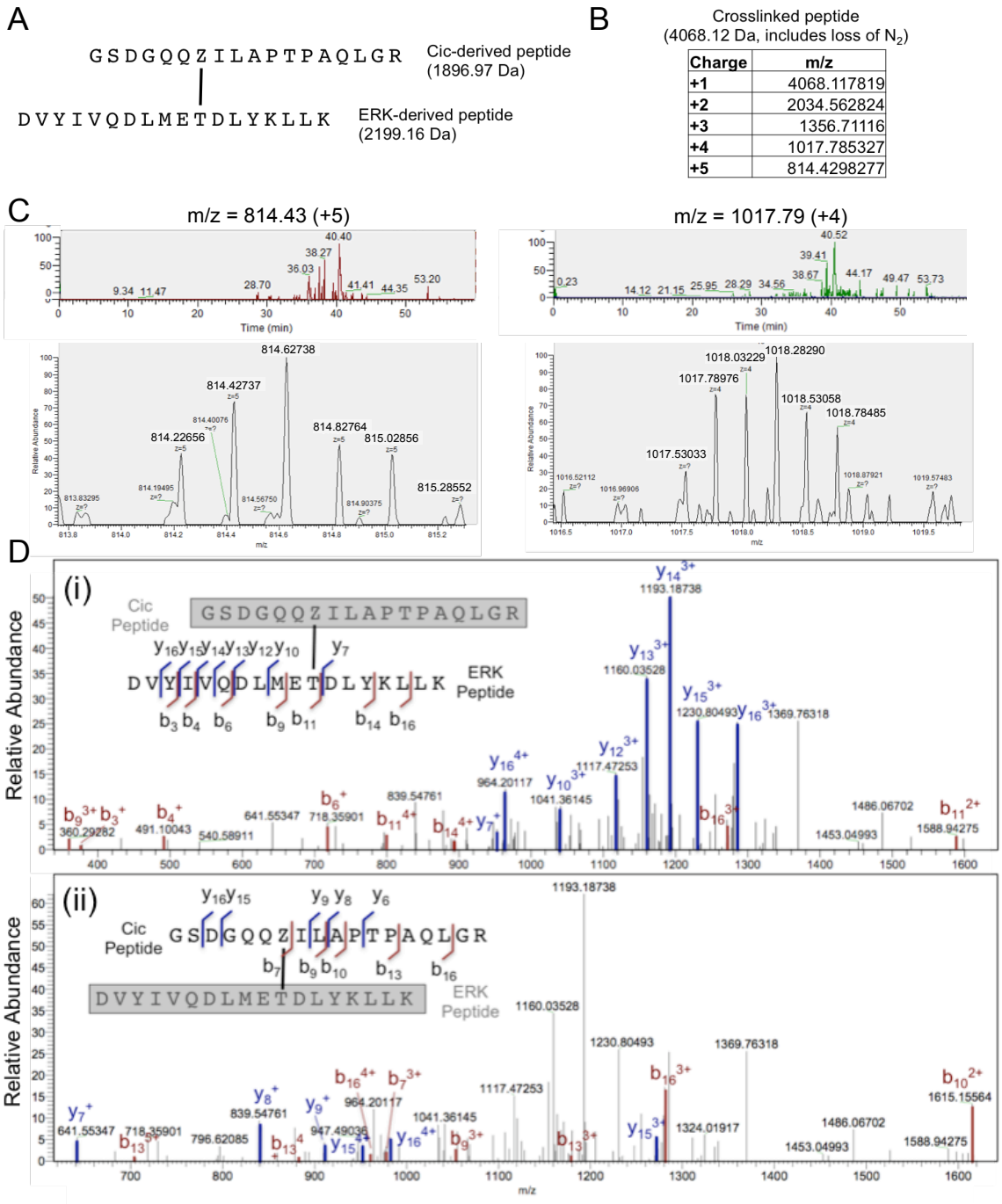

Fig. S4. Identification of ERK-*Drosophila* Cic crosslinked peptide by mass spectrometry. *(A)* Sequence and mass of Cic- and ERK-derived peptides that make up the crosslinked peptide. *(B)* Expected m/z of different charge states of the ERK-Cic crosslinked peptide. *(C)* Extracted ion chromatogram (top) and mass spectrum (bottom) of +5 and +4 charge states of the ERK-Cic crosslinked peptide. *(D)* Tandem mass spectrometry of the crosslinked ERK-Cic peptide. *(i)* *b*- and *y*- series fragments of the ERK peptide with a full-length Cic peptide adduct. *(ii)* *b*- and *y*-series fragments of the Cic peptide with a full-length ERK peptide adduct. *b*-series fragments are shown in red and *y*-series fragments are shown in blue.

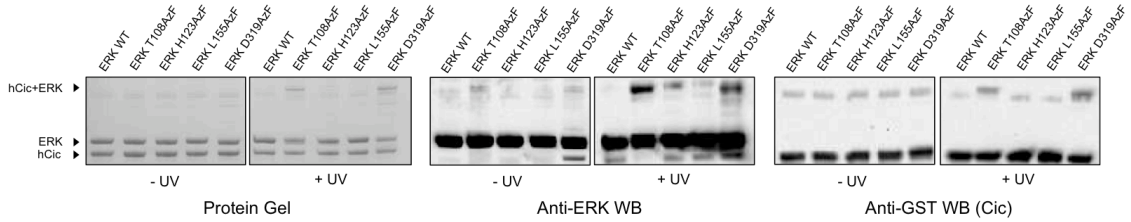

Fig. S5. Photocrosslinking of ERK AzF-incorporated variants and GSThCic. GSThCic was incubated with AzF-incorporated ERK variants and exposed to UV light. Crosslinking of GSThCic to ERK T108AzF and ERK D319AzF was confirmed by the UV-dependent appearance of a slow migrating band present in a protein gel, anti-ERK Western blot, and anti-GST Western blot (detects GSThCic).

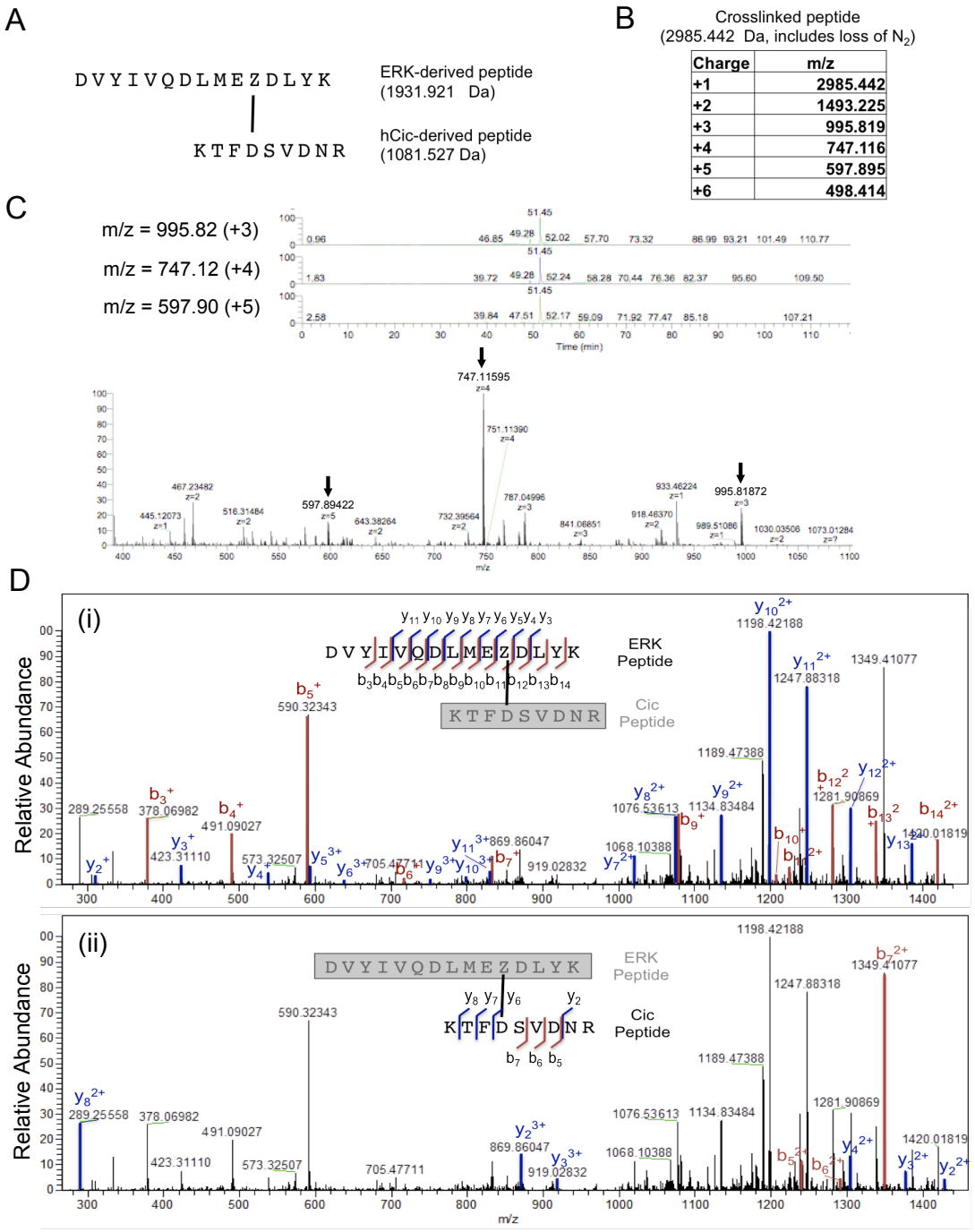

Fig. S6. Identification of ERK T108AzF-human Cic crosslinked peptide by mass spectrometry. *(A)* Sequence and mass of Cic- and ERK-derived peptides that make up the crosslinked peptide. *(B)* Expected m/z of different charge states of the ERK-Cic crosslinked peptide. *(C)* Extracted ion chromatogram (top) and mass spectrum (bottom) of +5, +4, and +3 charge states of the ERK-Cic crosslinked peptide (relevant peaks in spectrum indicated with an arrow). *(D)* Tandem mass spectrometry of the crosslinked ERK T108AzF-human Cic peptide. *(i) b-* and *y-* series fragments of the ERK peptide with a full-length Cic peptide adduct. *(ii) b-* and *y-* series fragments of the Cic peptide with a full-length ERK peptide adduct. *b*-series fragments are shown in red and *y*-series fragments are shown in blue.

ERK (fragments) +

hCic (full-length)

	b+	2	3		y+	2	3	
1	-	-	-	**D**	2985.4	1493.2	995.8	**15**
2	215.1	108.1	72.4	**V**	2870.4	1435.7	957.5	**14**
3	**378.2**	189.6	126.7	**Y**	2771.3	**1386.2**	924.5	**13**
4	**491.3**	246.1	164.4	**I**	2608.3	**1304.6**	870.1	**12**
5	**590.3**	295.7	197.4	**V**	2495.2	**1248.1**	**832.4**	**11**
6	**718.4**	359.7	240.1	**Q**	2396.1	**1198.6**	**799.4**	**10**
7	**833.4**	417.2	278.5	**D**	2268.1	**1134.5**	**756.7**	**9**
8	946.5	473.7	316.2	**L**	2153.0	**1077.0**	718.4	**8**
9	**1077.5**	539.3	359.8	**M**	2040.0	**1020.5**	680.7	**7**
10	**1206.6**	603.8	402.9	**E**	1908.9	955.0	**637.0**	**6**
11	2448.2	**1224.6**	816.7	**Z-hCic**	1779.9	890.4	**594.0**	**5**
12	2563.2	**1282.1**	855.1	**D**	**538.3**	269.6	180.1	**4**
13	2676.3	**1338.6**	892.8	**L**	**423.3**	212.1	141.8	**3**
14	2839.3	**1420.2**	947.1	**Y**	**310.2**	155.6	104.1	**2**
15	2985.4	1493.2	995.8	**K**	147.1	74.1	49.7	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S5. Expected m/z of *b*- and *y*-series fragments of ERK T108AzF peptide with full-length human Cic peptide adduct.

hCic (fragments) +

ERK (full-length)

	b+	2	3		y+	2	3	
1	-	-	-	**K**	2985.4	1493.2	995.8	**1**
2	202.1	101.6	68.1	**T**	2857.3	**1429.2**	953.1	**2**
3	349.2	175.1	117.1	**F**	2756.3	**1378.7**	919.4	**3**
4	2396.2	1198.6	799.4	**D-ERK**	2609.2	**1305.1**	870.4	**4**
5	2483.2	**1242.1**	828.4	**S**	590.3	295.6	197.4	**5**
6	2582.3	**1291.6**	861.4	**V**	503.3	252.1	168.4	**6**
7	2697.3	**1349.1**	899.8	**D**	404.2	202.6	135.4	**7**
8	2811.3	1406.2	937.8	**N**	**289.2**	145.1	97.1	**8**
9	2985.4	1493.2	995.8	**R**	175.1	88.1	59.0	**9**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S6. Expected m/z of *b*- and *y*-series fragments of human Cic peptide with full-length ERK T108AzF peptide adduct.

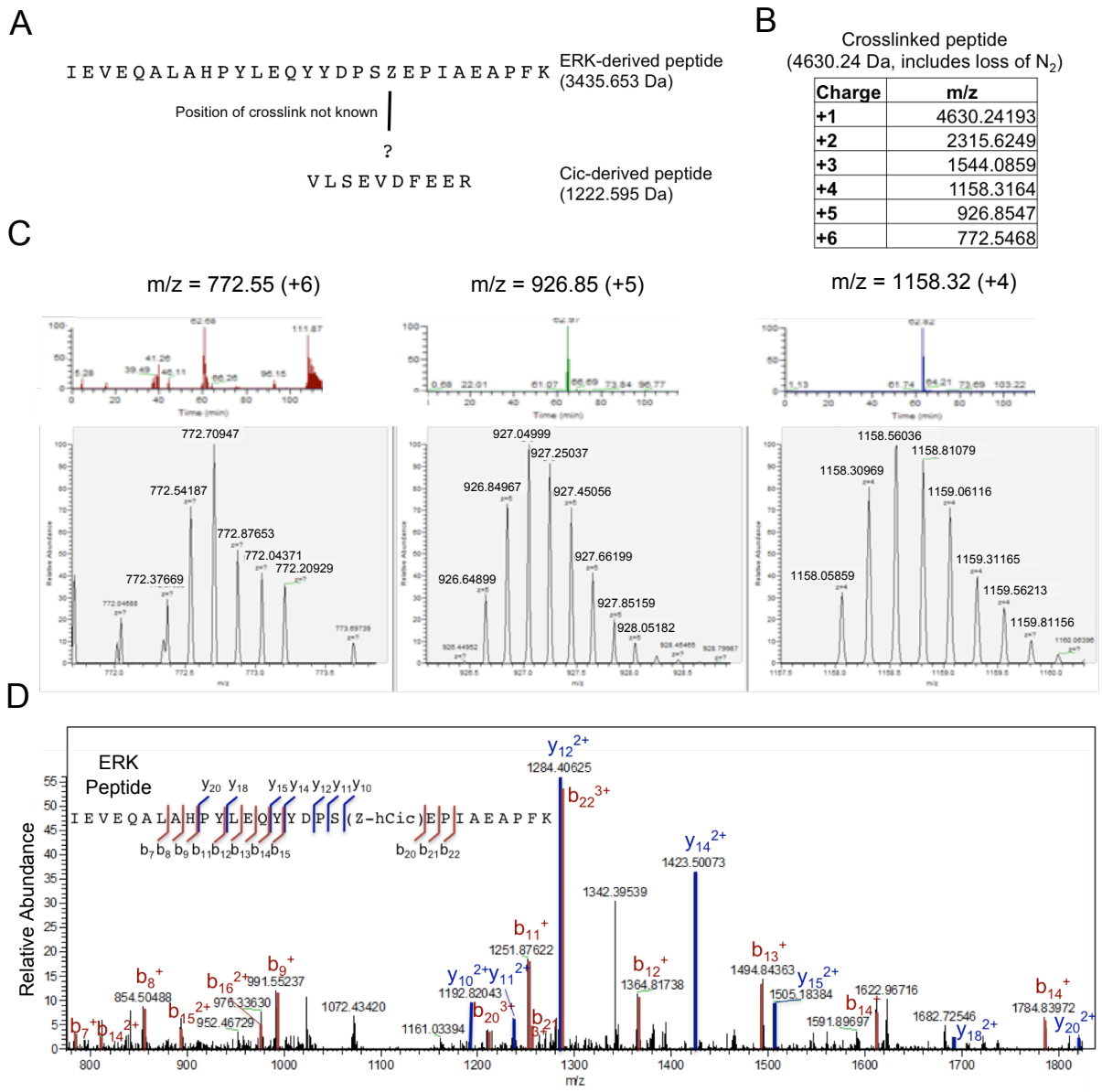

Fig. S7. Identification of ERK D319AzF-human Cic crosslinked peptide by mass spectrometry. *(A)* Sequence and mass of Cic- and ERK-derived peptides that make up the crosslinked peptide. *(B)* Expected m/z of different charge states of the ERK-Cic crosslinked peptide. *(C)* Extracted ion chromatogram (top) and mass spectrum (bottom) of +6, +5, and +4 charge states of the ERK-Cic crosslinked peptide. *(D)* Tandem mass spectrometry of the crosslinked ERK D319AzF-human Cic peptide. *y*- and *b*-series fragments of the ERK peptide with a full-length Cic peptide adduct are shown in blue and red, respectively. No fragments of Cic with a full-length ERK peptide adduct were found.

ERK (fragments) +

hCic (full-length)

	b+	2	3		y+	2	3	
1	-	-	-	**I**	4630.2	2315.6	1544.1	**29**
2	243.1	122.1	81.7	**E**	4517.2	2259.1	1506.4	**28**
3	342.2	171.6	114.7	**V**	4388.1	2194.6	1463.4	**27**
4	471.2	236.1	157.8	**E**	4289.1	2145.0	1430.4	**26**
5	599.3	300.2	200.4	**Q**	4160.0	2080.5	1387.3	**25**
6	670.3	335.7	224.1	**A**	4032.0	2016.5	1344.7	**24**
7	**783.4**	392.2	261.8	**L**	3960.9	1981.0	1321.0	**23**
8	**854.5**	427.7	285.5	**A**	3847.8	1924.4	1283.3	**22**
9	**991.5**	496.3	331.2	**H**	3776.8	1888.9	1259.6	**21**
10	1088.6	544.8	363.5	**P**	3639.7	**1820.4**	1213.9	**20**
11	**1251.6**	626.3	417.9	**Y**	3542.7	1771.8	1181.6	**19**
12	**1364.7**	682.9	455.6	**L**	3379.6	**1690.3**	1127.2	**18**
13	**1493.8**	747.4	498.6	**E**	3266.5	1633.8	1089.5	**17**
14	**1621.8**	**811.4**	541.3	**Q**	3137.5	1569.3	1046.5	**16**
15	**1784.9**	**893.0**	595.6	**Y**	3009.4	**1505.2**	1003.8	**15**
16	1948.0	**974.5**	650.0	**Y**	2846.4	**1423.7**	949.5	**14**
17	2063.0	1032.0	688.3	**D**	2756.3	1378.7	919.5	**13**
18	2160.0	1080.5	720.7	**P**	2568.3	**1284.6**	856.8	**12**
19	2247.1	1124.0	749.7	**S**	2471.2	**1236.1**	824.4	**11**
20	3629.7	1815.4	**1210.6**	**Z-hCic**	2384.2	**1192.6**	795.4	**10**
21	3758.8	1879.9	**1253.6**	**E**	1001.5	501.3	334.5	**9**
22	3855.8	1928.4	**1285.9**	**P**	872.5	436.8	291.5	**8**
23	3968.9	1985.0	1323.6	**I**	775.4	388.2	259.2	**7**
24	4039.9	2020.5	1347.3	**A**	662.4	331.7	221.5	**6**
25	4169.0	2085.0	1390.3	**E**	591.3	296.2	197.8	**5**
26	4240.0	2120.5	1414.0	**A**	462.3	231.6	154.8	**4**
27	4337.1	2169.0	1446.4	**P**	391.2	196.1	131.1	**3**
28	4484.1	2242.6	1495.4	**F**	294.2	147.6	98.7	**2**
29	4630.2	2315.6	1544.1	**K**	147.1	74.1	49.7	**1**

Bolded m/z values represent fragments identified in MS/MS spectrum

Table S7. Expected m/z of *b*- and *y*-series fragments of ERK D319AzF peptide with full-length human Cic peptide adduct.

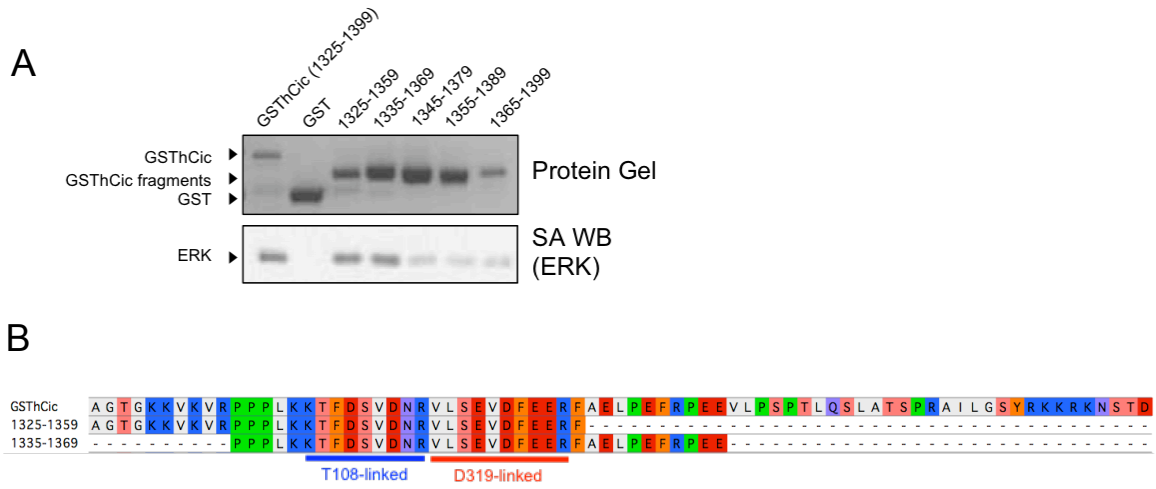

Fig. S8. Confirmation of ERK-binding region of GSThCic. (*A*) Thirty-five amino acid fragments of human Cic shifted by ten amino acids that span the 75 amino acids of human Cic present in GSThCic were tested for binding to ERK by GST pull-down. Two fragments (1325-1359 and 1335-1369) were found to bind to ERK. (*B*) The human Cic tryptic peptides that crosslink to ERK T108AzF (underlined in blue) and ERK D319AzF (underlined in red) are both present in the two fragments of GSThCic that bound to ERK in the GST pull-down.