

# Supporting Information

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1 MDYKDDDDKHNPEPGKKPVSAFNKKPRSAEVTAGSAAVFEAETERSGVKVRWQRDGS DIT  
61 ANDKYGLAAEGKRHTLTVRDA SPDDQGSYAVIAGSSKVKFDLKVTEPAPPEKAESEVAPG  
121 APKEVPAPATELEESVSSPEGSSVSVTQDGSAAEHQGAPDDPIGLFLMRPQDGEVTVGGSI  
181 VFSARVAGASLLKPPVVKWFKGKWDLSKVKVQHLQLHDSYDRASKVYLFELHITDAQTT  
241 SAGGYRCEVSTKDKFDS CNFNLTVHEAIGSGDLLRS AFRRTSLAGAGRRTSDSHEDAGT  
301 LDFSSLLKKRDSFRRDSKLEAPAEEDVWEILRQAPPSEYERIAFQHGVTDLRGMLKRLKG  
361 MKQDEKKS TAFQKKLEPAYQVNKGHKIRLTVELADPDAEVKWLKNGQEIQMSGSKYIFES  
421 VGAKR TLTI SQCSLADDAAYQCVVGGKECSTELFVKEPPVLI TRSLEDQLVMVGQVEFE  
481 CEVSEEGAQVKWLDGVELTREE TFKYRFKKDGRKHHLIINEATLEDAGHYAVRTSGGQS  
541 LAELIVQEKLEVYQSIADLAVGAKDQAVFKCEVSDENVRGVWLKNGKELVPDNRIKVVSH  
601 IGRVHKL TIDDVTPADEADYSFVPEGFACNLSAKLHFMEVKIDFVPRQEP PKIHLDCPGS  
661 TPD TIVVAGNKLRLDVPISGDPAPT VVWQKT V TQGKKA STGPHDPAPEDAGADEEWVFD  
721 KKLCE TEGRVRVETTKDRSVFTVEGAKEDEGVYTVTVKNPVGEDQVNLTVKVIDVPA  
781 PAAPKI SNVGEDSCTVQWEPPAYDGGQPV LGYILERK KKS YRWMRLNFDLLRELSHEAR  
841 RMIEGVAYEMRVYAVNAVGM SRP SPASQPFMPI GPPGEP THLAVEDVSDTTVSLKWRPPE  
901 RVGAGGLDGY SVEYCQEGCSEWTPALQGLTERTSMLVKDLP T GARLLFRVRAHNVAGPGG  
961 PIVTKEPVTVQEI LQRPRQLPRHLRQT IQKKVGEPVNLLI PFQGKPRPQVTWTKEGQPL  
1021 AGEEV SIRNSPTD TILFIRAARRTHSGTYQVTVRIENMEDKATLILQIVDKPSP PQDIRI  
1081 VETWGFNVALEWKPPQDDGNTEIWGYTVQKADKKTMEWFTVLEHYRRTHCVVSELIIGNG  
1141 YYFRVFSHNMVGS SSKAAATKEPVFI PRPGIT YEPPKYKALDFSEAPSFTQPLANRSIIA  
1201 GYNAILCCA VRGS PKPKI SWFKNGLDLGEDARFRMFCKQGVLTLEIRKPCPYDGGVYVCR  
1261 ATNLQGEAQCECRLEVRVPQ

Domains	Amino acid residues	Domains	Amino acid residues
Flag	1 - 10	C5	655 - 777
C0	11 - 104	Linker	778 - 780
Pro-Ala-rich linker	105 - 160	C6	781 - 874
C1	161 - 263	Linker	875
MyBP-C Motif	264 - 364	C7	876 - 973
C2	365 - 455	Linker	974 - 975
Linker	456 - 457	C8	976 - 1069
C3	458 - 546	Linker	1070
Linker	547 - 548	C9	1071 - 1166
C4	549 - 642	Linker	1167 - 1183
Linker	643 - 654	C10	1184 - 1280

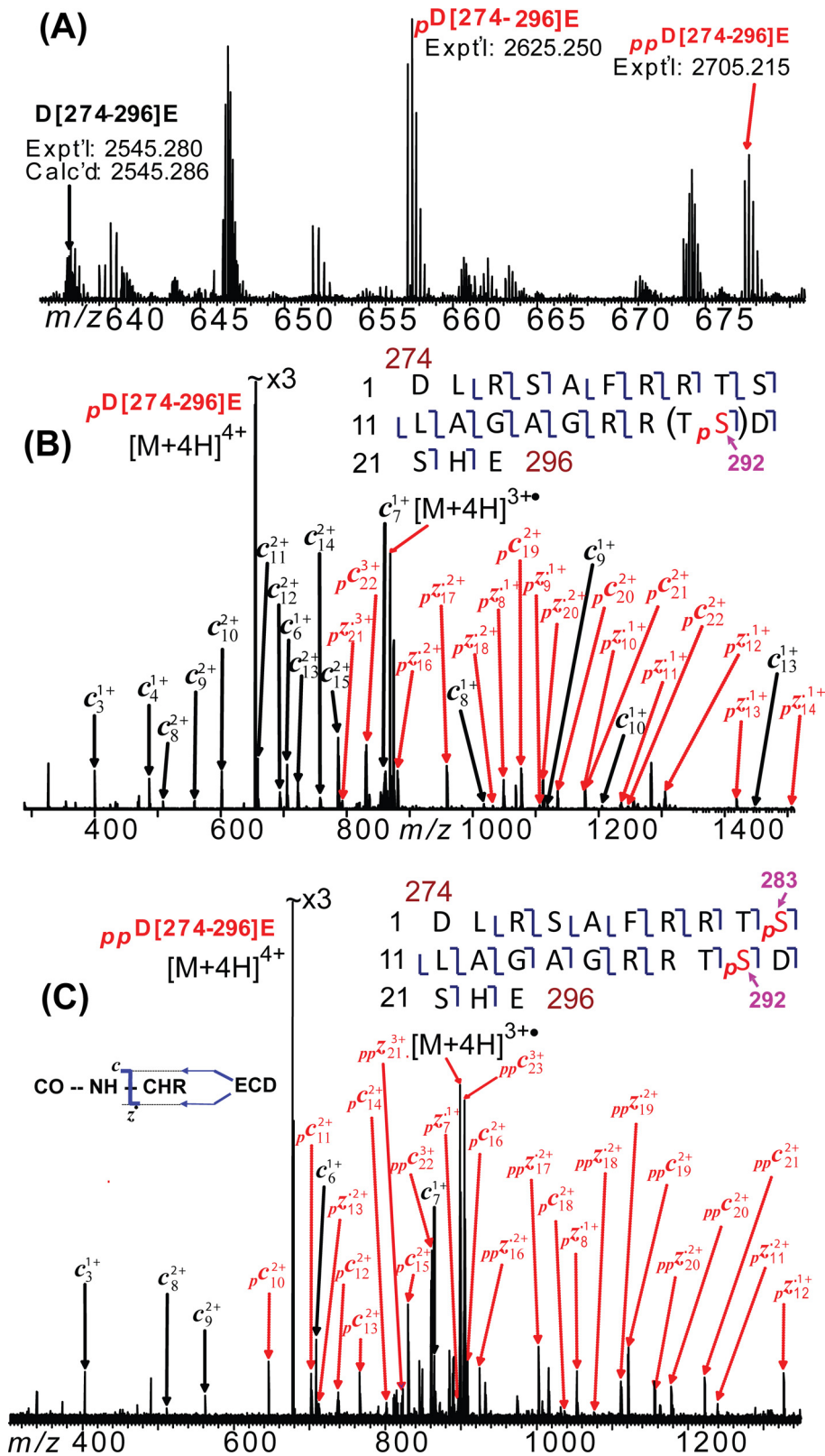
**Fig. S1.** Full-length recombinant cMyBP-C amino acid sequence and corresponding domain structure. Ser (S) and Thr (T) residues are highlighted in RED. S283, S292, and S312 in recombinant cMyBP-C correspond to the three potential phosphorylation sites in endogenous mouse cMyBP-C, S273, S282, and S302.











**Fig. S6.** Identification of Ser-283 and Ser-292 as phosphorylation sites from limited Asp-N proteolysis of full-length cMyBP-C. (A) Partial ESI/FTMS spectrum showing peptide D[274–296]E is mono- and bis-phosphorylated. (B) ECD of monophosphorylated D[274–296]E (pD[274–296]E) locates a phosphorylation site to Thr-291/Ser-292. (C) ECD of bisphosphorylated D[274–296]E (ppD[274–296]E) locates two phosphorylated sites to Ser-283 and Ser-292. The identified phosphorylation sites and the phosphorylated *c/z*\* fragmentation ions are labeled with a “p.”



**Table S1. Peptide assignments from limited Asp-N/Glu-C/Lys-C proteolysis of truncated cMyBP-C, C0-C4, expressed in baculovirus for localization of phosphorylation sites**

Peptide mass	Assignment	Error, Da	Mono-P	Bis-P
3484.689 <sup>a</sup>	M[1–30]E	0.021	ND	ND
4417.166 <sup>a</sup>	M[1–40]E	0.038	ND	ND
12613.350 <sup>a</sup>	M[1–116]E	0.106	ND	ND
5520.766 <sup>b</sup>	D[5–54]R	–0.022	ND	ND
5779.842 <sup>b</sup>	D[5–57]S	–0.027	ND	ND
6294.156 <sup>b</sup>	D[5–62]N	0.049	ND	ND
8190.203 <sup>b</sup>	D[5–79]R	0.063	ND	ND
5175.687 <sup>b</sup>	D[8–54]R	–0.021	ND	ND
2576.309 <sup>c</sup>	K[25–49]K	–0.010	ND	ND
2891.484 <sup>a</sup>	R[45–70]E	0.021	ND	ND
3172.538 <sup>a</sup>	G[56–85]D	0.000	ND	ND
3304.703 <sup>a</sup>	G[71–101]D	0.023	ND	ND
4366.286 <sup>a</sup>	G[71–111]E	0.031	ND	ND
4694.466 <sup>a</sup>	G[71–114]E	0.036	ND	ND
4910.543 <sup>a</sup>	G[71–116]E	0.039	ND	ND
2630.295 <sup>c</sup>	R[73–97]K	–0.010	ND	ND
2141.019 <sup>b</sup>	D[80–100]F	–0.008	ND	ND
3530.811 <sup>a</sup>	A[81–114]E	0.033	ND	ND
3746.878 <sup>a</sup>	A[81–116]E	0.036	ND	ND
4769.431 <sup>b</sup>	D[101–147]Q	0.065	ND	ND
3409.585 <sup>a</sup>	L[102–134]E	–0.165	ND	ND
13585.967 <sup>b</sup>	D[148–273]L	–0.195	ND	ND
2192.049 <sup>a</sup>	H[154–173]E	0.017	ND	ND
12565.621 <sup>b</sup>	D[159–273]L	–0.275	ND	ND
3158.618 <sup>a</sup>	A[267–296]E	0.025	D	ND
4805.486 <sup>a</sup>	A[267–311]D	0.031	D	ND
2077.117 <sup>b</sup>	D[274–292]S	–0.008	D	ND
4738.476 <sup>b</sup>	D[274–315]R	0.025	D	D
3694.891 <sup>a</sup>	A[298–329]E	0.033	D	ND
3909.958 <sup>b</sup>	D[316–349]T	0.041	ND	ND
5564.912 <sup>b</sup>	D[316–363]Q	0.046	ND	ND
2840.451 <sup>b</sup>	D[326–349]T	0.027	ND	ND
4495.407 <sup>b</sup>	D[326–363]Q	0.034	ND	ND
2926.592 <sup>a</sup>	R[341–365]E	0.018	ND	ND
3568.958 <sup>b</sup>	D[364–394]A	–0.015	ND	ND
11556.063 <sup>b</sup>	D[364–467]E	–0.107	ND	ND
3140.801 <sup>a</sup>	K[366–392]E	0.025	ND	ND
13927.12 <sup>c</sup>	K[367–491]K	–0.030	D	ND
3188.653 <sup>b</sup>	D[468–494]K	0.024	D	ND
2145.113 <sup>b</sup>	D[495–511]K	–0.009	ND	ND
3872.066 <sup>b</sup>	D[495–526]E	0.033	ND	ND
2429.359 <sup>a</sup>	T[504–522]E	0.015	ND	ND
3698.885 <sup>c</sup>	H[516–549]K	–0.016	ND	ND
3459.832 <sup>b</sup>	D[527–558]A	0.032	ND	ND
2254.149 <sup>a</sup>	V[553–573]E	0.020	ND	ND
4517.493 <sup>b</sup>	D[576–615]A	0.032	ND	ND
4832.606 <sup>b</sup>	D[576–618]A	0.038	ND	ND
3136.720 <sup>a</sup>	L[590–617]E	0.018	ND	ND
4045.124 <sup>a</sup>	L[590–625]E	0.034	ND	ND
2100.182 <sup>b</sup>	D[593–610]D	–0.009	ND	ND
3059.470 <sup>b</sup>	D[616–642]I	0.029	ND	ND
2744.358 <sup>b</sup>	D[619–642]I	0.024	ND	ND
9902.465 <sup>c</sup>	L[635–641]K	–0.004	ND	ND
1015.548 <sup>c</sup>	L[635–642]I	–0.004	ND	ND

Peptide masses listed here are monoisotopic masses. Superscript a, b, and c denote Glu-C, Asp-N, and Lys-C proteolysis, respectively. "Mono-P" and "bis-P" stand for mono- and bis-phosphorylated forms. "ND" is short for "not detected" and "D" is for "detected".



**Table S2. Peptide assignments from Asp-N/Glu-C/Lys-C proteolysis of full-length cMyBP-C (C0-C10) expressed in baculovirus for localization of phosphorylation sites**

Peptide mass	Assignment	Error, Da	Mono-P	Bis-P
6100.021 <sup>b</sup>	M[1–54]R	–0.003	ND	ND
8769.365 <sup>b</sup>	M[1–79]R	–0.012	ND	ND
15741.56 <sup>b</sup>	M[1–147]Q	+0.081	ND	ND
6700.272 <sup>b</sup>	D[2–62]N	–0.020	ND	ND
6294.096 <sup>b</sup>	D[5–62]N	–0.011	ND	ND
15064.530 <sup>b</sup>	D[5–147]Q	0.024	ND	ND
15740.770 <sup>b</sup>	D[8–158]P	–0.087	ND	ND
2576.322 <sup>c</sup>	K[25–49]K	0.003	ND	ND
3121.541 <sup>a</sup>	T[43–70]E	–0.013	ND	ND
2159.074 <sup>a</sup>	R[45–63]D	–0.009	ND	ND
2891.451 <sup>a</sup>	R[45–70]E	–0.012	ND	ND
4037.056 <sup>b</sup>	D[63–100]F	–0.003	ND	ND
3304.666 <sup>a</sup>	G[71–101]D	–0.014	ND	ND
4910.483 <sup>a</sup>	G[71–116]E	–0.021	ND	ND
2630.300 <sup>c</sup>	R[73–97]K	–0.005	ND	ND
2141.025 <sup>b</sup>	D[80–100]F	–0.002	ND	ND
4769.359 <sup>b</sup>	D[101–147]Q	–0.007	ND	ND
5789.784 <sup>b</sup>	D[101–158]P	–0.008	ND	ND
1803.918 <sup>a</sup>	V[117–134]E	–0.007	ND	ND
7565.825 <sup>c</sup>	E[124–198]K	0.010	ND	ND
2421.246 <sup>b</sup>	D[148–170]Q	0.108	ND	ND
7529.985 <sup>b</sup>	D[148–218]H	0.039	ND	ND
13357.750 <sup>b</sup>	D[148–271]G	0.089	ND	ND
13585.786 <sup>b</sup>	D[148–273]L	0.008	ND	ND
12337.075 <sup>b</sup>	D[159–271]G	–0.201	ND	ND
12565.580 <sup>b</sup>	D[159–273]L	0.234	ND	ND
1852.920 <sup>c</sup>	V[211–226]K	0.002	ND	ND
3810.813 <sup>b</sup>	D[219–252]K	–0.003	ND	ND
1690.903 <sup>b</sup>	D[222–235]T	–0.001	ND	ND
3835.884 <sup>b</sup>	D[222–255]F	0.000	ND	ND
1821.824 <sup>a</sup>	L[232–248]E	–0.007	ND	ND
2162.987 <sup>b</sup>	D[236–255]F	–0.003	ND	ND
2082.958 <sup>a</sup>	V[249–266]E	–0.010	ND	ND
3230.610 <sup>b</sup>	D[272–301]L	–0.004	D	D
2545.280 <sup>b</sup>	D[274–296]E	–0.006	D	D
3002.500 <sup>b</sup>	D[274–301]L	–0.003	D	D
4077.117 <sup>b</sup>	D[274–310]S	–0.004	D	D
4738.444 <sup>b*</sup>	D[274–315]R	–0.006	D	D
2018.005 <sup>b</sup>	D[293–310]R	–0.002	ND	ND
2211.174 <sup>b</sup>	D[297–315]R	–0.002	D	ND
1092.628 <sup>b</sup>	D[302–310]R	–0.001	ND	ND
1753.957 <sup>b</sup>	D[302–315]R	–0.002	D	ND
5668.903 <sup>c</sup>	K[309–356]K	0.007	D	ND
1087.502 <sup>b</sup>	D[316–325]E	–0.001	ND	ND
5564.857 <sup>b</sup>	D[316–363]Q	–0.009	ND	ND
4393.222 <sup>c</sup>	L[319–356]K	0.006	ND	ND
4495.373 <sup>b</sup>	D[326–363]Q	0.000	ND	ND
1672.958 <sup>b</sup>	D[350–363]Q	–0.001	ND	ND
21969.250 <sup>b</sup>	D[364–558]A	–0.043	ND	ND
22123.540 <sup>c</sup>	S[368–565]K	0.137	ND	ND
1301.630 <sup>a</sup>	I[409–419]E	–0.003	ND	ND
1012.519 <sup>c</sup>	Y[416–424]K	–0.004	ND	ND
3380.607 <sup>b</sup>	D[437–467]E	–0.092	ND	ND
5774.776 <sup>c</sup>	E[457–506]K	–0.141	ND	ND
2958.552 <sup>a</sup>	T[504–527]D	–0.032	ND	ND
3698.907 <sup>c</sup>	H[516–549]K	0.006	ND	ND
1717.794 <sup>a</sup>	D[527–543]E	–0.008	ND	ND
3574.648 <sup>a</sup>	D[527–559]D	–0.178	ND	ND
1778.885 <sup>b</sup>	D[559–574]S	–0.002	ND	ND
1828.935 <sup>a</sup>	V[574–589]E	–0.008	ND	ND
1952.046 <sup>b</sup>	D[576–592]P	–0.002	ND	ND
4517.457 <sup>b</sup>	D[576–615]A	–0.004	ND	ND
4832.564 <sup>b</sup>	D[576–618]A	–0.004	ND	ND

Peptide mass	Assignment	Error, Da	Mono-P	Bis-P
15763.74 <sup>b</sup>	D[576–719]F	0.070	ND	ND
4615.421 <sup>b</sup>	D[616–655]L	0.134	ND	ND
2054.081 <sup>b</sup>	D[656–675]L	–0.002	ND	ND
2090.155 <sup>c</sup>	L[673–691]K	0.003	ND	ND
3097.616 <sup>b</sup>	D[676–705]P	–0.003	ND	ND
3509.774 <sup>b</sup>	D[676–709]E	–0.005	ND	ND
3823.893 <sup>b</sup>	D[676–713]A	–0.008	ND	ND
2941.491 <sup>b</sup>	D[682–709]E	–0.002	ND	ND
3255.615 <sup>b</sup>	D[682–713]A	–0.001	ND	ND
4060.934 <sup>b</sup>	D[682–719]F	–0.010	ND	ND
2568.130 <sup>c</sup>	K[698–721]K	–0.010	ND	ND
2440.048 <sup>c</sup>	A[699–721]K	0.003	ND	ND
2104.129 <sup>b</sup>	D[720–737]K	–0.002	ND	ND
3551.822 <sup>b</sup>	D[720–750]E	–0.033	ND	ND
12349.901 <sup>b</sup>	D[720–829]F	–0.418	ND	ND
1238.604 <sup>c</sup>	E[750–760]K	0.001	ND	ND
1605.786 <sup>b</sup>	D[751–765]E	–0.003	ND	ND
1411.732 <sup>c</sup>	N[761–773]K	0.001	ND	ND
1438.758 <sup>a</sup>	Q[767–779]D	–0.045	ND	ND
2644.235 <sup>b</sup>	D[779–803]Y	0.023	ND	ND
3647.772 <sup>c</sup>	I[786–818]K	–0.002	ND	ND
3181.436 <sup>b</sup>	D[804–829]F	–0.297	ND	ND
8457.276 <sup>c</sup>	S[821–895]K	0.059	ND	ND
4755.246 <sup>c</sup>	W[896–938]K	–0.006	ND	ND
4409.430 <sup>a</sup>	R[932–972]E	–0.018	ND	ND
2811.602 <sup>c</sup>	D[939–965]K	0.004	ND	ND
3175.845 <sup>c</sup>	E[966–991]K	0.003	ND	ND
2731.564 <sup>c</sup>	K[992–1015]K	0.011	ND	ND
5184.647 <sup>c</sup>	E[1016–1061]K	0.010	ND	ND
10771.510 <sup>c</sup>	E[1016–1110]K	–0.010	ND	ND
17222.593 <sup>a</sup>	I[1078–1229]E	–0.030	ND	ND
14726.410 <sup>a</sup>	G[1099–1229]E	–0.048	ND	ND
3939.892 <sup>a</sup>	I[1103–1134]E	–0.063	ND	ND
2130.143 <sup>c</sup>	E[1162–1179]K	–0.008	ND	ND
11007.56 <sup>b</sup>	D[1182– 1280]Q	0.078	ND	ND
2770.358 <sup>b</sup>	D[1230–1252]Y	–0.065	ND	ND
1465.663 <sup>a</sup>	G[1254–1267]E	–0.035	ND	ND
2977.569 <sup>a</sup>	G[1254– 1280]Q	–0.137	ND	ND

Peptide masses listed here are monoisotopic masses. <sup>a</sup>, <sup>b</sup>, <sup>c</sup>, <sup>d</sup> denote Glu-C, Asp-N, and Lys-C proteolysis respectively. "Mono-P" and "bis-P" stand for mono- and bis-phosphorylated forms. "ND" is short for "not detected" and "D" is for "detected".

\*Mono, bis- and Tris-phosphorylations were detected.