Calcineurin Selectively Docks With The Dynamin Ixb Splice Variant To Regulate Activity-Dependent Bulk Endocytosis

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Supplementary Data

Tables 1-3. *Identification of CaN catalytic and regulatory subunits and calmodulin by mass spectrometry.*
Tandem mass spectrometry data from LC-MSMS analysis of SDS-PAGE Coomassie stained bands matched to protein sequences. The mascot.dll 1.6vb21 script was used to export data to Mascot v2.2 (Matrix Science, UK) and the database was the International Protein Index Rat v3.54 (39925 sequences; 20363545 residues). Peptides with a Mascot score less than 35 were excluded. Peptides are separated into lists of common and isoform specific sequences. The percentage of the protein sequence covered by the mass spectrometry data is shown below each Table. Oxidised Met and deamidated Asn/Gln are underlined.

Supplementary Table 1. *CaN catalytic subunit identification by MS from the 58 kDa protein in Fig 1B.*

Tryptic peptide and adjacent residues	**Sequence Position**	**Precursor m/z and Error (ppm)**	**Charge**	**Mascot Score**	**Mascot Expect**
Peptides specific to alpha isoforms (1 and 2):					
K.LSTTDRVVK.A	11-19	509.8 (-17)	2+	39	0.027
K.AVPFPPSHR.L	20-28	504.3 (-20)	2+	42	0.01
R.LTAKEVFDNDGKPR.V	29-42	530.6 (6)	3+	58	0.00024
K.EVFDNDGKPR.V	33-42	588.8 (0)	2+	53	0.001
R.LEESVALR.I	56-63	458.8 (-12)	2+	62	0.00014
R.IITEGASILR.Q	64-73	536.8 (-26)	2+	75	0.000063
R.IITEGASILRQEK.N	64-76	486.6 (10)	3+	51	0.00014
K.TLFLLR.G	143-148	381.7 (-13)	2+	43	0.0089
K.TQEHFTHNTVR.G	244-254	457.2 (-3)	3+	47	0.0031
K.GLTPTGMLPSGVLSGGK.Q	425-441	794.4 (-7)	2+	90	0.0000001
K.ITSFEEAK.G	457-464[2] 467-474[1]	462.7 (-68)	2+	42	0.009
K.ITSFEEAKGLDR.I	457-468[2] 467-478[1]	455.9 (-9)	3+	48	0.0026
R.RDAMPSDANLNSINK.A	477-491[2] 487-501[1]	554.6 (5)	3+	57	0.00035
R.DAMPSDANLNSINK.A	478-491[2] 488-501[1]	753.8 (-4)	2+	78	0.0000025
Peptides common to both alpha (1 and 2) and beta isoforms:					
K.LFEVGGSPANTR.Y	101-112[α] 110-121[β]	624.3 (-72)	2+	70	0.00005
R.YLFLGDYVDR.G	113-122[α] 122-131[β]	630.8 (-12)	2+	63	0.000087
K.SQTTGFPSLITIFSAPNYLDVYNNK.A	294-318[α] 303-327[β]	930.8 (14)	3+	40	0.0013
K.YENNVMNIR.Q	324-332[α] 333-341[β]	584.8 (-12)	2+	61	0.00013
R.EESESVLTLK.G	415-424[α] 425-434[β]	567.8 (-7)	2+	50	0.0018
Peptides specific to alpha isoform 1:					
K.QTLQSATVEAIEADEAIK.G	442-459	640.0	3+	54	0.00064

		(11)			
Peptides specific to alpha isoform 2:					
K.QTLQSAIK.G	442-449	444.8 (-31)	2+	64	0.000088
Peptides specific to beta isoform:					
R.IINEGAAILR.R	73-82	535.3 (-15)	2+	66	0.000035
K.GLTPTGMLPSGVLAGGR.Q	435-451	800.4 (-20)	2+	52	0.0011
R.QTLQSATVEAIEAEK.A	452-466	639.7 (-3)	3+	38	0.025

Calcineurin catalytic subunit alpha Isoform 1: 38% coverage

```
  1 MSEPKAIDPK LSTTDRVVKA VPFPPSHRLT AKEVFDNDGK PRVDILKAHL
 51 MKEGRLEESV ALRIITEGAS ILRQEKNLLD IDAPVTVCGD IHGQFFDLMK
101 LFEVGGSPAN TRYLFLGDYV DRGYFSIECV LYLWALKILY PKTLFLLRGN
151 HECRHLTEYF TFKQECKIKY SERVYDACMD AFDCLPLAAL MNQQFLCVHG
201 GLSPEINTLD DIRKLDRFKE PPAYGPMCDI LWSDPLEDFG NEKTQEHFTH
251 NTVRGCSYFY SYPAVCDFLQ HNNLLSILRA HEAQDAGYRM YRKSQTTGFP
301 SLITIFSAPN YLDVYNNKAA VLKYENNVMN IRQFNCSPHP YWLPNFMDVF
351 TWSLPFVGEK VTEMLVNVLN ICSDDELGSE EDGFDGATAA ARKEVIRNKI
401 RAIGKMARVF SVLREESESV LTLKGLTPTG MLPSGVLSGG KQTLQSATVE
451 AIEADEAIKG FSPQHKITSF EEAKGLDRIN ERMPPRRDAM PSDANLNSIN
501 KALASETNGT DSNGSNSSNI Q
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Calcineurin catalytic subunit alpha Isoform 2: 36% coverage

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  1 MSEPKAIDPK LSTTDRVVKA VPFPPSHRLT AKEVFDNDGK PRVDILKAHL
 51 MKEGRLEESV ALRIITEGAS ILRQEKNLLD IDAPVTVCGD IHGQFFDLMK
101 LFEVGGSPAN TRYLFLGDYV DRGYFSIECV LYLWALKILY PKTLFLLRGN
151 HECRHLTEYF TFKQECKIKY SERVYDACMD AFDCLPLAAL MNQQFLCVHG
201 GLSPEINTLD DIRKLDRFKE PPAYGPMCDI LWSDPLEDFG NEKTQEHFTH
251 NTVRGCSYFY SYPAVCDFLQ HNNLLSILRA HEAQDAGYRM YRKSQTTGFP
301 SLITIFSAPN YLDVYNNKAA VLKYENNVMN IRQFNCSPHP YWLPNFMDVF
351 TWSLPFVGEK VTEMLVNVLN ICSDDELGSE EDGFDGATAA ARKEVIRNKI
401 RAIGKMARVF SVLREESESV LTLKGLTPTG MLPSGVLSGG KQTLQSAIKG
451 FSPQHKITSF EEAKGLDRIN ERMPPRRDAM PSDANLNSIN KALASETNGT
501 DSNGSNSSNI Q
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Calcineurin catalytic subunit beta Isoform: 20% coverage

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  1 MAAPEPARAA PPPPPPPPPP LGADRVVKAV PFPPTHRLTS EEVFDMDGIP
 51 RVDVLKNHLV KEGRVDEEIA LRIINEGAAI LRREKTMIEV EAPITVCGDI
101 HGQFFDLMKL FEVGGSPANT RYLFLGDYVD RGYFSIECVL YLWVLKILYP
151 STLFLLRGNH ECRHLTEYFT FKQECKIKYS ERVYEACMEA FDSLPLAALL
201 NQQFLCVHGG LSPEIHTLDD IRRLDRFKEP PAFGPMCDLL WSDPSEDFGN
251 EKSQEHFSHN TVRGCSYFYN YPAVCEFLQN NNLLSIIRAH EAQDAGYRMY
301 RKSQTTGFPS LITIFSAPNY LDVYNNKAAV LKYENNVMNI RQFNCSPHPY
351 WLPNFMDVFT WSLPFVGEKV TEMLVNVLSI CSDDELMTEG EDQFDVGSAA
401 ARKEIIRNKI RAIGKMARVF SVLREESESV LTLKGLTPTG MLPSGVLAGG
451 RQTLQSATVE AIEAEKAIRG SSPPHRICSF EEAKGLDRIN ERMPPRKDAV
501 QQDGFNSLNT AHTTENHGTG NHSAQ
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Supplementary Table 2. *Calcineurin subunit B (regulatory) isoform 1 and/or 2 (all peptides are common to both isoforms) identification by LC-MSMS from the 19 kDa protein in Fig 1C.*

Tryptic peptide and adjacent residues	Sequence Position	Precursor m/z and Error (ppm)	Charge	Mascot Score	Mascot Expect
K.KLDLDNSGSLSVEEFMSLPELQQNPLVQR.V	29 - 57	826.7 (41)	4+	57	0.00032
K.LDLDNSGSLSVEEFMSLPELQQNPLVQR.V	30 - 57	1058.86 (0)	3+	77	0.0000024
K.EFIEGVSQFSVK.G	74 - 85	685.34 (-17)	2+	73	0.0000079
R.IYDMDKDGYISNGELFQVLK.M	98 - 117	788.71 (-6)	3+	82	0.000001
K.MMVGNNLKDTQLQQIVDK.T	118 - 135	703.34 (-8)	3+	43	0.0079
K.DTQLQQIVDK.T	126 - 135	594.32 (11)	2+	66	0.000043
K.TIINADKDGDGR.I	136 - 147	425.55 (1)	3+	37	0.038

Calcineurin subunit B regulatory isoform 1: 53% coverage

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  1 MGNEASYPLE MCSHFDADEI KRLGKRFKKL DLDNSGSLSV EEFMSLPELQ
 51 QNPLVQRVID IFDTDGNGEV DFKEFIEGVS QFSVKGDKEQ KLRFAFRIYD
101 MDKDGYISNG ELFQVLKMMV GNNLKDTQLQ QIVDKTIINA DKDGDGRISF
151 EEFCAVVGGL DIHKKMVVDV
```

Supplementary Table 3. *Calmodulin identification by LC-MSMS from the 21 kDa protein in Fig 1C.*

Tryptic peptide and adjacent residues	Sequence Position	Precursor m/z and Error (ppm)	Charge	Mascot Score	Mascot Expect
K.EAFSLFDKDGDGTITTK.E	15 - 31	615.63 (-6)	3+	91	0.0000002
K.ELGTVMR.S	32 - 38	411.21 (-12)	2+	40	0.02
K.DTDSEEEIR.E	79 - 87	547.23 (-7)	2+	49	0.002
K.DTDSEEEIREAFR.V	79 - 91	532.91 (-8)	3+	44	0.0057
R.VFDKDGNGYISAAELR.H	92 - 107	585.62 (-9)	3+	54	0.00054
R.EADIDGDGQVNYEEFVQMMTAK.-	128 - 149	841.36 (-4)	3+	54	0.00049

Calmodulin: 50% coverage

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  1 MADQLTEEQI AEFKEAFSLF DKDGDGTITT KELGTVMRSL GQNPTEAELQ
 51 DMINEVDADG NGTIDFPEFL TMMARKMKDT DSEEEIREAF RVFDKDGNGY
101 ISAAELRHVM TNLGEKLTDE EVDEMIREAD IDGDGQVNYE EFVQMMTAK
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Supplementary Figure Legends

Supplementary Fig S1: CaN binding to dynIxb-PRD increases in the presence of Ca^{2+}. Synaptosomal lysates were incubated with GST-dynI-PRD (either xa or xb) coupled to GSH-sepharose beads in the presence of 0.2 mM Ca^{2+}. Bound proteins were separated by SDS-PAGE and stained with Coomassie blue. The protein bound to dynIxb-PRD at about 58 kDa (A), 19 kDa (B), and 21 kDa(B), were identified by MS as the A and B subunits of protein phosphatase 2B (calcineurin, CaNA, and CaNB) and calmodulin (CaM).

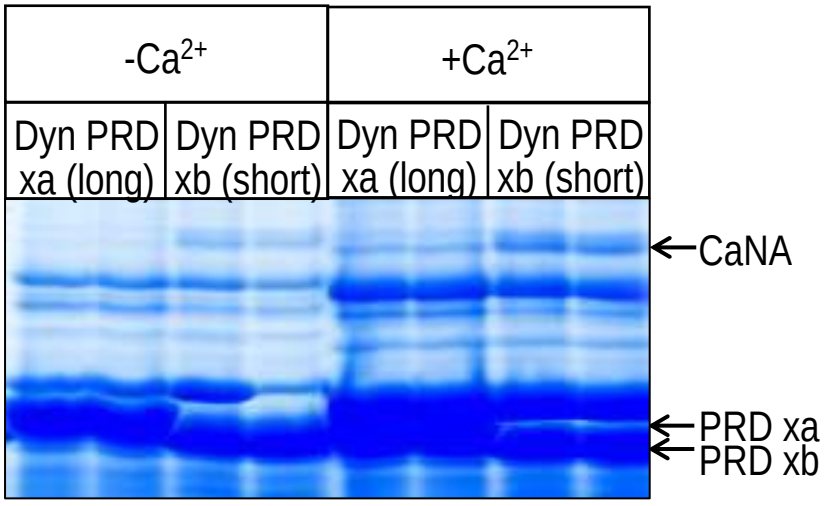
Supplementary Fig S2: Mapping the CaN binding site within the dynIxb-PRD. (**A**) The penetratin-tagged peptide mimetic of the short variant, $dynIxb_{842-851}$ was synthesized and used for competition studies. Synaptosomal lysates were incubated with GST-dynIxb-PRD immobilized on GSH-sepharose beads in the absence or presence of 5-250 μM of the peptide, and in presence of 0.2 mM Ca^{2+}. Binding of CaN and CaM was detected by western blot analysis with anti-CaNA, CaNB, and CaM antibodies. (**B**) CaN binding to GST-dynIxb-PRD is unaffected by pseudo-phosphorylation at the phosphobox. Pull-down experiments were performed with GST-DynIxb-PRD mutated at Ser-774 and Ser-778 in its phospho-box region. Both phospho-deficient mutations of the serines to alanine or phospho-mimetic mutations to glutamic acid were utilised. CaN binding was analysed by western blot with anti-CaNA antibodies. (**C**) CaN binding to GST-dynIxb-PRD is blocked by prebinding of syndapin to PRD. His-tagged full-length syndapin expressed in bacterial cell lysates was incubated with GST-DynIxb-PRD coupled GSH-sepharose beads for 1 hour. These syndapin bound GST-DynIxb-PRD beads were used to pull down proteins from synaptosomal lysate. CaNA binding was analysed by western blot with anti-CaNA antibodies. All results in A-C are representative of two independent experiments.

Supplementary Fig S3: The CaN-dynIxb interaction does not regulate KCl-evoked dephosphorylation of three other dephosphins, synaptojanin (SJ), AP180, and amphiphysin I. (**A**) Rat brain synaptosomes were metabolically labelled with ^{32}Pi, washed, and incubated with or without either 100 μM PRITIS peptide, or 30 μM CsA for 15 min. Synaptosomes were then stimulated with 41 mM KCl for 10 s. After lysis, synaptojanin was pulled-down with GST-amphiphysin I-SH3 bound to GSH-sepharose beads, and separated by SDS-PAGE. Protein in the 160 kDa region showing SJ visualized by Coomassie blue staining demonstrates even sample loading (A, upper panel). Phosphorylated SJ was visualized by autoradiography of quadruplicate samples (A, lower panel). (**B**) Following the pull-down from Fig. 3, two additional dephosphins, AP180 and amphiphysin I, were extracted by a second sequential pull-down with GST-α-adaptin-ear domain bound to GSH-sepharose beads of the same lysates. Proteins in the 120 kDa and 180 kDa regions showing amphiphysin I and AP180 individually visualized by Coomassie blue staining demonstrate even sample loading (B, upper panel). Phosphorylated amphiphysin I and AP180 were visualized by autoradiography of triplicate samples (B, lower panel). (**C**) Rat brain synaptosomes were labelled with ^{32}Pi, washed, and incubated with or without either 100 μM PRITIS, 100 μM ARATAS peptide, or 30 μM CsA for 15 min. After lysis, dynIxa and xb were pulled-down with GST-amphiphysin I-SH3 bound to GSH-sepharose beads, and separated by a 12% SDS-PAGE containing 0.08% bis-arylamide (C, upper panel), phosphorylated dynIxa and xb were visualized by autoradiography of triplicate samples (C, lower panel). (D) AP180 and amphiphysin I were extracted and visualized as (B), upper panel is Coomassie blue stained

gel showing even sample loading, and lower panel is an autoradiograph of phosphorylated proteins from triplicate samples. All results are representative of least two independent experiments.

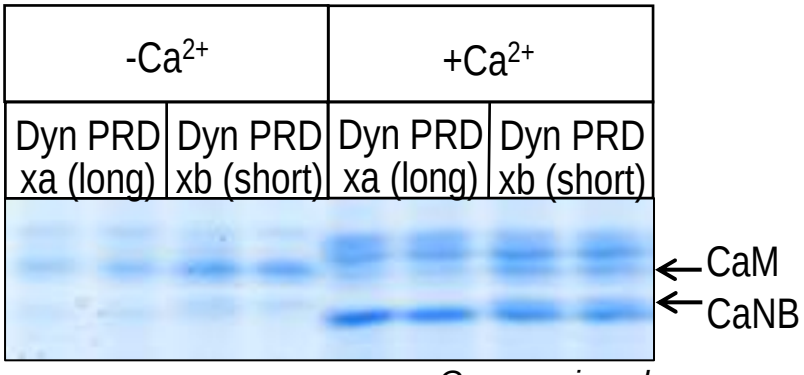
Supplementary Fig S4: DynIxa and xb are present in cerebellar granule neurons. (**A**) 14-day-old primary cultured rat brain cerebellar granule neurons were lysed and the lysate was incubated with GST-AmpI-SH3 coated GSH-sepharose beads. Bound proteins were separated by a 12% SDS-PAGE containing 0.08% bis-acrylamide at pH 9.2 {{20615}} and visualized by Coomassie blue staining, and protein bands were excised from the gel and identified by LC-MS/MS (Obitrap Velos mass spectrometry). (B) Dynamin I was either pulled down by GST-amphiphysin I-SH3 bound to GSH-sepharose beads or immunoprecipitated (IP) with antisera against dynamin I (Hudy-1) from cerebellar granule neuron lysates. Bound proteins were analysed by western blot and the first lane shows a sample of the input lysate prior to pull down. All results are representative of least two independent experiments.

A

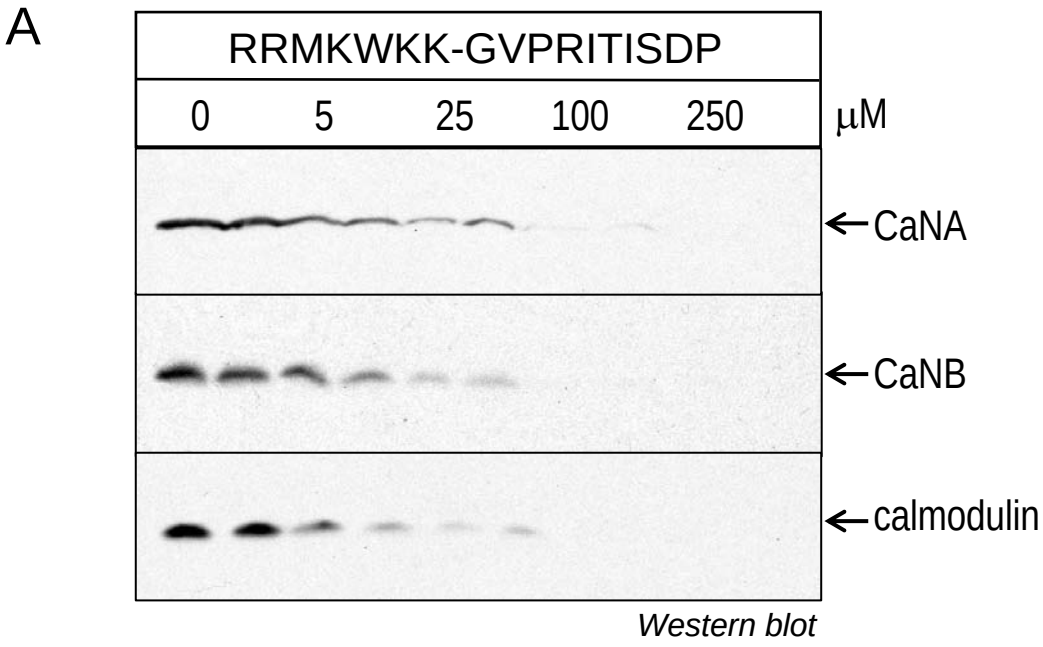


Coomassie gel

B



Coomassie gel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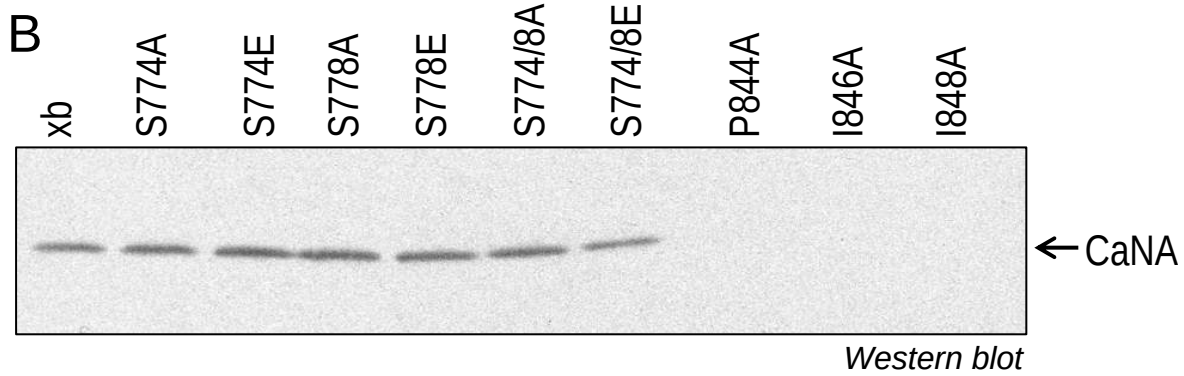
0
5
25
100
250
μM
CaNA
CaNB
calmodulin
Western blot


B
xb
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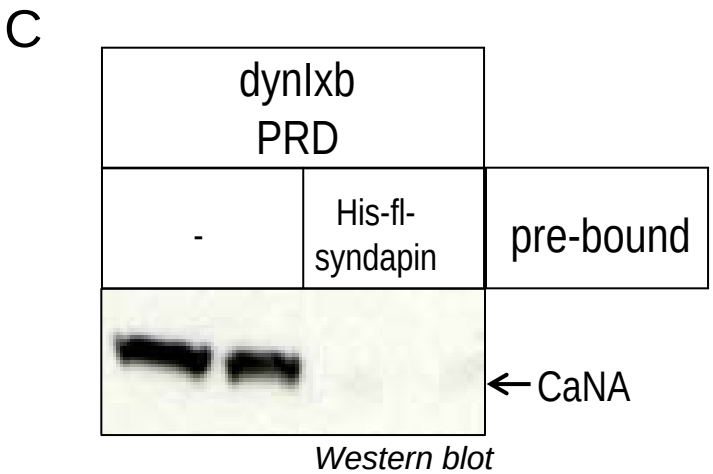
CaNA
Western blot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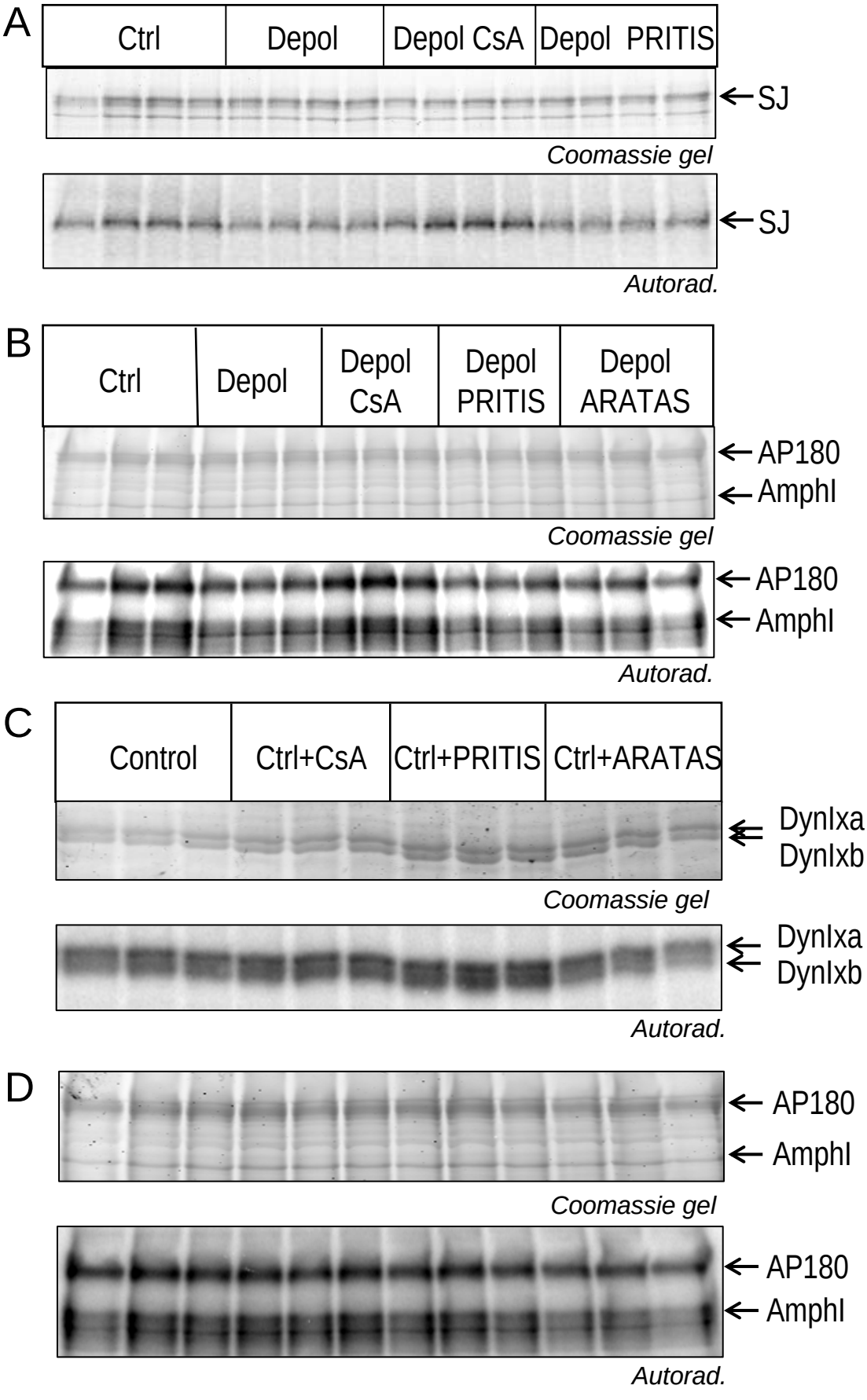
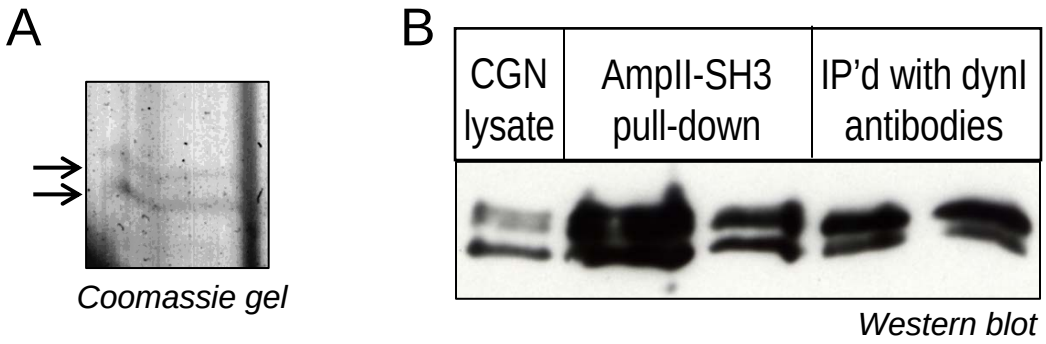
dynIxb
PRD
-
His-fl-
syndapin
pre-bound
CaNA
Western blot



Suppl Fig. S3 – Xue et al.



Suppl Fig. S4 – Xue et al.