Supplemental information

"Individual Carboxypeptidase D domains have both redundant and unique functions in *Drosophila* development and behavior"

Galyna Sidyelyeva, Christian Wegener, Brian P. Schoenfeld, Aaron J. Bell, Nicholas E. Baker, Sean M. J. McBride, and Lloyd D. Fricker

Contents:

Methods

Creation of transgenic lines

Constructs: All of the following cDNA constructs were verified by nucleotide sequence analysis. To produce the constructs uas:svr1A-2-3t2 and uas:svr1B-2-3t2, we used EST clones LP12324 and LD2840 to obtain the 5'utr for the 1A and 1B CPD forms respectively. PCR (Invitrogen platinum Taq polymerase) was utilized to obtain a 320 bp (1A) and a 340 bp band (1B), both flanked with BamH1 and NotI sites on the 5' end, which were ligated (Quick ligation kit, NEB) into the BamH1/AflII sites for 1A and into the BamH1/XbaI sites for 1B into the expression constructs 1A/wt and 1B/wt, described in (Sidyelyeva et al., 2006), resulting in the constructs named fCPD1A5'utr and fCPD1B5'utr. CP domain 3 and 3'utr 1.95 kb band was obtained by RT-PCR (One Step SSIII HiFi, Invitrogen), using RNA from wild type Drosophila (Qiagen RNAeasy kit) and ligated into the SalI/XhoI sites of pBluescript, resulting in the construct named GS-D3. Then 2.6 kb bands containing 5'utr and 1A/wt or 1B/wt were cut from the fCPD1A5'utr and fCPD1B5'utr constructs and ligated into the NotI/SalI sites of GS-D3 plasmid, resulting in the cDNA1A and cDNA1B constructs. Full length 4.6 kb bands containing 1A-2-3t2 or 1B-2-3t2 were then ligated into the NotI/XhoI sites of the pUAST vector (Brand and Perrimon, 1993).

To obtain the uas:svr1B-2-3t1 construct, CPD domain 3 clones in pCR4-TOPO vectors were digested with SacI/BamHI enzymes and screened for the 430 bp tail1 band. The correct plasmid was propagated in SCS110 dam$^-$ strain (Stratagen) as well as the cDNA1B plasmid to allow the cloning of the 450 bp tail1 band using BclI and XhoI sites. The 4.6 kb band was ligated into the NotI/XhoI sites of the pUAST plasmid, resulting in the uas:svr1B-2-3t1 construct.

The uas:svr1A-short and uas:svr1B-short constructs were made by PCR amplification (platinum Taq HiFi, Invitrogen) from EST clones LP12324 and LD28490 respectively. For both constructs 5'EcoRI and 3'KpnI restriction sites were introduced during PCR. 1A-short 1.5 kb and 1B-short 1.6 kb bands were subcloned into the pCRII-TOPO vector for sequencing, and then ligated into the EcoRI/KpnI sites of the pUAST vector.

The uas:svr1B-2-t2 construct was created using the cDNA1B construct. CP domain 3 containing 1.5 kb band was cut out using AatII and HpaI. The adaptor of 200 bp which contains CPD bridge 2 and HA tag introduced by PCR was subcloned into the pCRII-TOPO vector for sequencing and then ligated into the AatII/HpaI sites of the cDNA1B plasmid, resulting in the cDNA1BdelCP3 construct. The svr1B-2-t2 construct was ligated into the NotI/XhoI sites of the pUAST vector.

The uas:svr2-3t2 construct was created from the cDNA1B construct. CP domain 1B 1.6 kb band was cut using XbaI and BglII. A 400 bp adaptor representing the signal peptide and CPD bridge 1 sequences was amplified by PCR (platinum Pfx polymerase, Invitrogen), subcloned into the pCRII-TOPO vector for sequencing and then ligated into the XbaI/BglII sites of the cDNA1B plasmid. The svr2-3t2 construct was ligated into the NotI/XhoI sites of the pUAST vector.

The uas:svr1B-2-1At2 and uas:svr1B-2-1Bt2 constructs were created using the cDNA1BdelCP3 construct. The cDNA1BdelCP3 plasmid was linearized with NdeI and HpaI restriction enzymes; CP domain 1A and 1B 1.2 kb bands were amplified using Roche HiFi Expand plus kit, subcloned into the pCRII-TOPO vector and sequenced. Then 1A or 1B domains were ligated into the NdeI/HpaI sites of cDNA1BdelCP3. The svr:1B-2-1At2 and svr:1B-2-1Bt2 constructs were cloned into the NotI/XhoI sites of the pUAST vector. The P-element mediated transformation was used to transform w^{1118} flies, performed by Best Gene Inc Company, California, USA.

Immunostaining

D.mel-2 cells were transfected with either uas:svr1B-2-t2 or uas:svr1B-2-1A-t2 or uas:svr1B-2-1Bt2 plasmid containing the HA tag (described above) and pBRAcpA constitutively expressing Gal4 (kindly provided by Dr. J. Backer) using electroporation (Gene Pulser, BioRad). After three days cells were fixed with 4% PFA in PBS, pH7.4 and stained with 1:1000 dilutions of antiserum against *Drosophila cis* Golgi (Abcam) and 1:2500 dilution of antiserum against HA tag (Sigma). Images were collected using a confocal microscope (Leica) and analyzed with ImageJ 1.40g (NIH) and Adobe Photoshop software.

Third instar larval central nervous system was dissected in phosphate buffer, pH 7.4, and fixed in 3.7% formalin in 0.1 M PIPES (pH 7.0), 2 mM MgSO4, 1 mM EGTA and stained with 1:200 dilution of an antiserum against GFP. Images were collected using a confocal microscope (BioRad) and analyzed with ImageJ 1.40g (NIH) and Adobe Photoshop software.

CPD purification and Western Blot

CPD from wild-type, 1Bshort and 1B-2-3-t2 flies was purified on p-aminobenzoyl-Arg-Sepharose affinity resin as described (Sidyelyeva et al., 2006). The initial extraction buffer did not contain detergent so that only soluble CPD forms were extracted and applied onto the columns. Columns were washed and then eluted first with high pH buffer (pH 8.0 Tris) to remove proteins that bind in a pH-dependent manner (like carboxypeptidase E) and then eluted with 25 mM Arg in Tris pH 8.0 to remove proteins that bind specifically to the Arg residue at both low and high pH. After fractioning on 4-20% polyacrylamide precast gel (Bio-Rad), proteins were transferred to a nitrocellulose membrane and probed with a 1:1000 dilution of polyclonal rabbit antiserum raised against a peptide corresponding to the N-terminus of the 1B form (see (Sidyelyeva et al., 2006). Bound antiserum was detected using a 1:5000 dilution of IRDye 800 conjugated α-rabbit IgG (Rockland-Inc) followed by analysis using an Odyssey® v1.2 Infrared Imaging System (LI-COR Biosciences).

Supplemental Tables

Table S1. Temperature dependent expression of the *uas:svr*1B-2-3-t2 transgene compared to wild type CPD expression.

Mutant line	Temperature, °C	Relative CPD mRNA levels
wt	18	1.0
wt	25	1.0
wt	30	0.9
1B-2-3-t2	18	22
1B-2-3-t2	25	72
1B-2-3-t2	30	101

Table S2. Relative transgenic CPD mRNA levels.

Mutant line	Temperature, °C	Relative CPD mRNA levels
wt	25	1.0
1B-2-3-t2	25	72
1B-2-3-t1	25	30
1A-2-3-t2	25	66
1B-short	25	47
2-3-t2	25	17
1B-2-t2	25	56
1B-2-1A-t2	25	53
1B-2-1B-t2	25	59
svr^{1}	25	2.6
svr^{poi}	25	6.9

Table S3. Survival of *svr^{PG33}; uas:svr* mutants

UAS:svr construct	Number of transgenic lines per transgene	18°C: low Gal4 expression			25°C: medium Gal4 expression		
		females	FM7 males	ywPG33;uas: svr males	females	FM7 males	ywPG33;uas: svr males
1A-2-3-t2	7	45	20	17	52	21	28
1B-2-3-t1	8	371	131	128	256	106	103
1B-2-3-t2	2	61	22	21	253	112	117
1A-short	8	49	22	0	58	26	0
1B-2-t2	9	260	104	24	186	64	28
2-3-t2	7	136	42	19	171	79	57
1B-short	9	207	71	7	353	138	84
1B-2-1A-t2	9	212	72	7	290	135	65
1B-2-1B-t2	9	254	75	12	322	122	78

Data are presented as the total number of flies for all lines for each individual transgene construct.

Supplemental figures

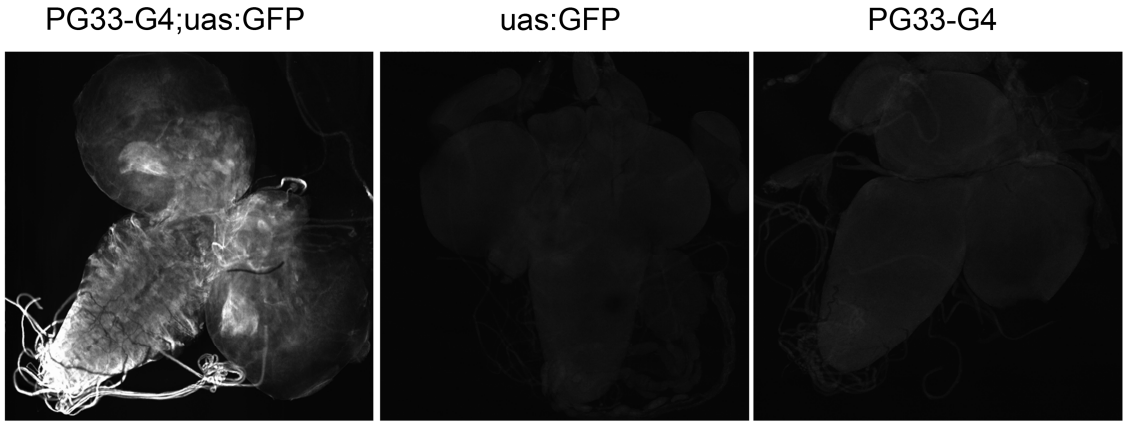

Figure S1. The GFP reporter, driven by the PG33-Gal4 promoter, shows broad distribution in the *Drosophila* central nervous system of the third instar larva. Confocal stacks of whole brain are shown.

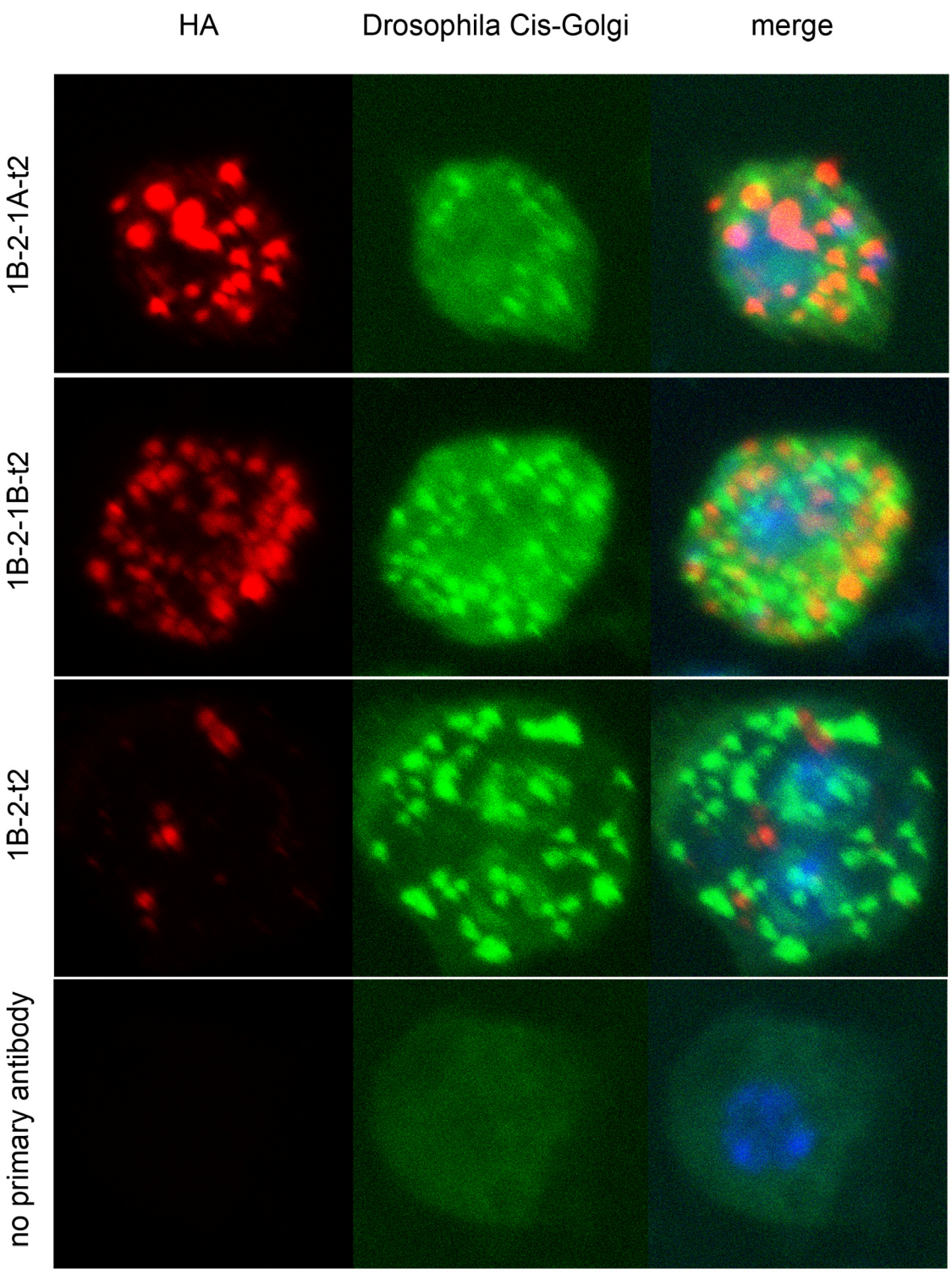

Figure S2. Cellular localization of HA-tagged uas:svr construct with altered third CP domain. Confocal stacks of the whole cells are shown. Because there was no commercially available *Drosophila* trans-Golgi marker, we used anti-GM130 antiserum as a marker for *Drosophila* cis-Golgi.

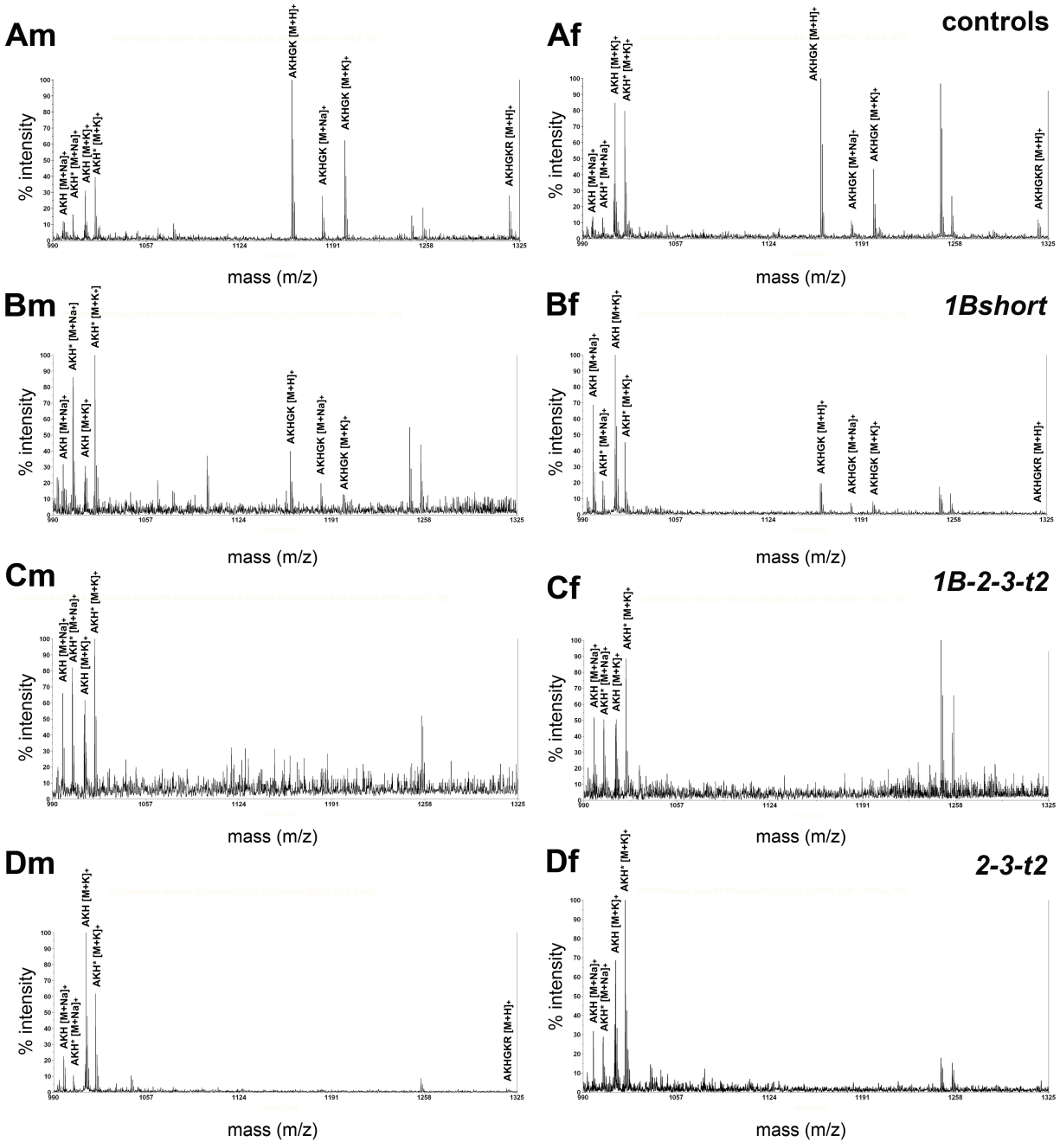

Figure S3. Quantitative direct mass spectrometric profiling of AKH and processing intermediates in the corpora cardiaca of transgenic CPD mutants. UAS:svr was used as control. Representative spectra are shown. Am-Dm = adult male flies, Af-Df = adult female flies. AKH* indicates the heavy isotope-labeled AKH that was used as internal standard. Typical for AKH, the $[M+Na]^+$ and $[M+K]^+$, but not the $[M+H]^+$ adduct is detectable.

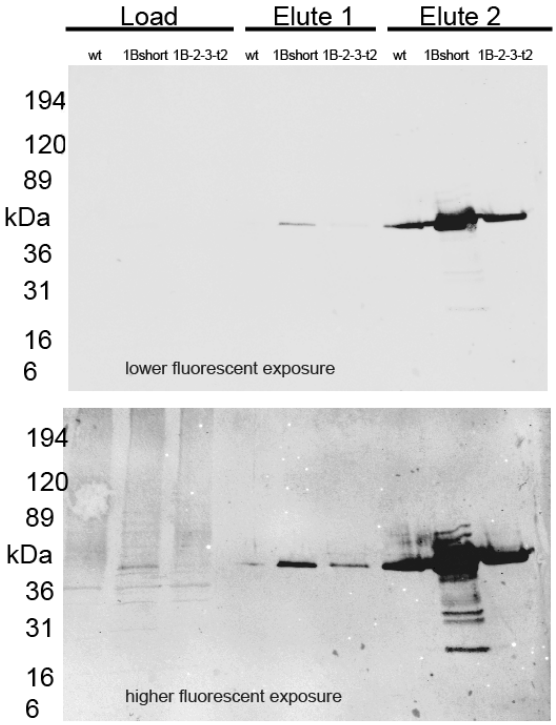

Figure S4. CPD long form is enzymatically processed into smaller forms. Soluble CPD protein from wild type, 1Bshort and 1B-2-3-t2 lines was extracted and purified on a para-aminobenzoyl-Arg affinity column and probed with antiserum raised against the 1B-N-terminus. Bound antiserum was detected by the near infrared fluorescence method (Odyssey, LI-COR Biosciences). The band at ~50 kDa corresponds to the soluble short form of CPD. The transgenic line 1Bshort shows CPD overexpression compared to wild type CPD flies. The finding that the transgenic line for the full-length form (1B-2-3-t2) shows a ~50 kDa protein in the soluble extracts suggests that proteolytic processing converts the long ~150-180 kDa form into the ~50 kDa soluble form. Molecular masses and the positions of markers are indicated. Load indicates the protein extract applied on affinity column. Elute 1 is the first elute, reprenting a pH 8.0 wash. Most of the CPD protein is expected to be in Elute 2, which contains 25 mM Arg and not in Elute 1. The high pH step elutes carboxypeptidase E-like enzymes that have an acidic pH optimum but not CPD which has a broader pH optimum and requires Arg to displace it from the resin, as described [20,21].