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Cytochrome *c* heme lyase can mature a fusion peptide composed of the amino-terminal residues of horse cytochrome *c*

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Electronic Supplementary Information

Synthesis of the PSD95-PDZ3 domain gene

The gene coding for residues 309-401 corresponding to the third PDZ domain (PDZ3) of the *Rattus norvegicus* post synaptic density 95 protein (PSD-95)[1-2] flanked between *Age*I and *Kpn*I restriction sites (Figure S1) was synthesized and inserted into a pMK cloning vector by GeneArt. The PDZ3 gene was designed to include an ochre stop codon before the *Kpn*I site for expression purposes. The PDZ3 gene was removed from the pMK vector using an *Nde*I site upstream from the PDZ3 gene and the *Kpn*I site and inserted into a pET17b plasmid (Novagen), yielding pETPDZ3. A second *Age*I site within the structural gene of PDZ3 (Figure S1) was removed from pETPDZ3 by making a G322G silent mutation (ACCGGT to ACCGGC) using the G322G-primers (Table S2) and Quick-Change site-directed mutagenesis methods (Stratagene). The resulting vector, pETPDZ3-1, contained the PDZ3 structural gene flanked between unique *Age*I and *Kpn*I sites.

		R	E	P	R	R	I	V	I	H	R	G	S	T	G	L	G	F	N	309-326
ACC	GGT	CGT	GAA	CCG	CGT	CGT	ATT	GTT	ATT	CAT	CGT	GGT	TCA	ACC	GGT	CTG	GGT	TTT	AAT	
Age I														*Age I*						
I	V	G	G	E	D	G	E	G	I	F	I	S	F	I	L	A	G	G	P	327-346
ATT	GTT	GGT	GGT	GAA	GAT	GGC	GAA	GGC	ATT	TTT	ATT	AGC	TTT	ATT	CTG	GCA	GGC	GGT	CCG	
A	D	L	S	G	E	L	R	K	G	D	Q	I	L	S	V	N	G	V	D	347-366
GCT	GAT	CTG	AGC	GGT	GAA	CTG	CGT	AAA	GGT	GAT	CAG	ATT	CTG	AGC	GTT	AAT	GGT	GTT	GAT	
L	R	N	A	S	H	E	Q	A	A	I	A	L	K	N	A	G	Q	T	V	367-386
CTG	CGT	AAT	GCA	AGC	CAT	GAA	CAG	GCA	GCA	ATT	GCA	CTG	AAA	AAT	GCA	GGT	CAG	ACC	GTT	
T	I	I	A	Q	Y	K	P	E	E	Y	S	R	F	E	Stop					387-401
ACC	ATT	ATC	GCA	CAG	TAT	AAA	CCG	GAA	GAA	TAT	AGC	CGC	TTT	GAA	TAA	GGT	AAC			
																Kpn I				

Figure S1. 5'-3' DNA sequence of the *Rattus norvegicus* PSD95-PDZ3 gene synthesized and contained in a pMK-cloning vector. Each codon of the gene contains the coding amino acid above, and the amino acid numbering (309-401) of PDZ3 in PSD95 is listed to the right. The restriction sites used or modified for this study are shown.

Construction of expression vectors.

The pBTR(hCc)[3] plasmid (Figure S2A) coding for horse heart cytochrome *c* (cyt *c*) and yeast heme lyase (CCHL) (Figure S2B) was a gift from Prof. Gary J. Pielak (University of North Carolina Chapel Hill). A *lac* promoter upstream of the gene cluster controls the expression of both cyt *c* and CCHL, and a gene coding β-lactamase for ampicillin resistance (*Amp*^{r}) is used for bacterial colony selection.

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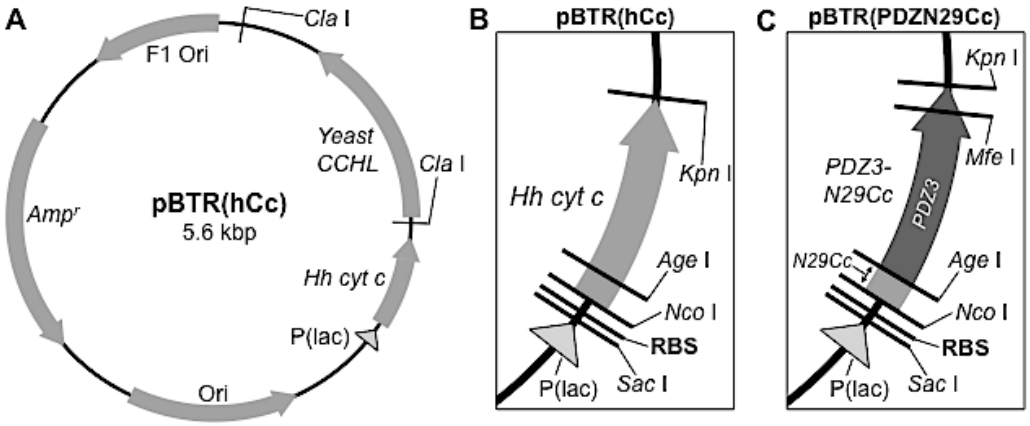

Figure S2. (**A**) pBTR(hCc) vector used as the template for construction of the PDZ-fusion expression vectors. (**B**) Expanded region of pBTR(hCc) showing the gene segment for horse heart cyt *c* (*Hh cyt c*) and (**C**) of pBTR(PDZN29Cc) showing the gene segment of PDZ3N29Cc. In pBTR(hCc), the lac promoter (P(lac)) controls transcription of the *Hh cyt c* and *yeast CCHL* genes, *Amp*^{r} codes for a β-lactamase for bacterial colony selection, F1 Ori and Ori are origins of replication, and the ribosomal binding site (RBS) controls translation of *Hh cyt c*. The unique restriction sites used to construct the vectors coding the PDZ-fusions are shown in each panel.

Table S1. Expression vectors used in this study.

Expression Vector	Description
pBTR(PDZN29Cc)	Codes for PDZ-N29Cc and CCHL
pBTR(PDZN18Cc)	Codes for PDZ-N18Cc and CCHL
pBTR(PDZC23Cc)	Codes for PDZ-C23Cc and CCHL
pBTR(-CCHL)	pBTR(PDZN29Cc) with CCHL gene removed
pBTR(PDZ3)	Wild type PDZ3 (contains no fusion tag)
pBTR(hCc)	Codes for horse cyt *c* and CCHL

Expression vectors constructed for this study are listed in Table S1 and are all derived from pBTR(hCc). Recombinant DNA manipulations and sub-cloning methods were followed according to standard protocol.[4] XL1-Blue *Escherichia coli* (Stratagene) was used for sub-cloning purposes. The correct nucleic acid sequence of all plasmid constructs was confirmed by DNA sequencing (University of Rochester Functional Genomics Center, Rochester, NY). pBTR(PDZN29Cc) coding for PDZ3 fused with residues 1-29 of cyt *c* was constructed by first digesting pBTR(hCc) with AgeI and KpnI to remove the gene segment coding for residues 30-104 of cyt *c*. The resulting vector, pBTR(N29Cc), was isolated by standard agarose gel purification methods (Qiagen). The PDZ3 structural gene was isolated and gel purified from pETPDZ3-1 using the same restriction sites and sub-cloned into pBTR(N29Cc), resulting in the final pBTR(PDZN29Cc) plasmid.

pBTR(PDZN18Cc) was constructed by removing the gene segment coding for the N29Cc fusion from pBTR(PDZN29Cc) using *Nco*I and *Age*I restriction sites and replacing it with the N18Cc DNA insert (IDT-DNA, Table S2) coding for N18Cc followed by a 5 residue linker, resulting in the complete pBTR(PDZ18hCc) vector. The single-stranded DNA insert coding for N18Cc was PCR amplified using the primers N18Cc-P1 and -P2 (Table S2) before the sub-cloning procedure.

To construct pBTR(PDZ3) containing wild-type PDZ3 without a fusion tag, pBTR(PDZN29Cc) was digested with SacI and AgeI to remove the N29Cc sequence and the DNA upstream of the ribosome binding site (RBS) (Figure S2C). A SacI-AgeI DNA insert (IDT-DNA, Table S2) coding for the DNA removed between *Sac*I and the start codon contained within the *Nco I* site of pBTR(PDZN29Cc) was PCR amplified using the SacI-AgeI-P1 and -P2 primers (Table S2) and sub-cloned into the gel purified and digested vector using the same restriction sites, resulting in pBTR(PDZ3). The PDZ3 expressed from this vector contains two additional residues, TG, at the N-terminus of the protein.

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To construct pBTR(PDZC23Cc), pBTR(PDZ3) was digested with MfeI and KpnI removing the gene segment coding for last several C-terminal residues of PDZ3 (Figure S2C). The DNA insert C23Cc (IDT-DNA, Table S2) coding for the region between *Mfe*I and *Kpn*I of pBTR(PDZ3) followed by DNA coding for the first 23 residues of cyt *c* (constituting the C23Cc tag) was sub-cloned into the gel purified and digested vector using the same restriction sites, resulting in pBTR(C23Cc).

pBTR(-CCHL) was constructed by removing the gene coding yeast CCHL from pBTR(PDZN29Cc) using the two *Cla*I sites in the vector. The gel-purified and digested vector was relegated using T4 DNA ligase to produce pBTR(-CCHL).

Table S2. Nucleic acid sequence of the DNA inserts and primers used to construct the vectors used in this study

Insert or primer	5'-3' DNA Sequence
G322G Primer 1	CATCGTGGTTCAACCGGCCTGGGTTTTAATATTGTTGG
G322G Primer 2	CCAACAATATTAAAACCCAGGCCGGTTGAACCACGATG
N18Cc Insert	CGTGCATCCATGGGCGACGTGGAAAAAGGCAAAAAGATCTTCGTGCAGAAATGCGCG CAGTGCCACGGCAGCGGCACCGGTCTGACG
N18Cc Primer 1	CGTGCATCCATGGGCGACGTGGAAAAAGGC
N18Cc Primer 2	CGTCAGACCGGTGCCGCTGCCGTGGCACTG
SacI-AgeI Insert	CGTGCAGAGCTCGTTACCCGGGGATCCATAACTAACTAATCTAGAATTAATTTTGTTTA ACTTTAAGAAGGAGATATATCCATGGGCACCGGTCTGACG
SacI-AgeI Primer 1	CGTGCAGAGCTCGTTACCCGGGGATCC
SacI-AgeI Primer 2	CGTCAGACCGGTGCCCATGGATATATCTCC
C23Cc Insert	CGTGCAGCAATTGCACTGAAAAATGCAGGTCAGACCGTTACCATTATCGCACAGTATA TAAACCGGAAGAATATAGCCGCTTTGAAGGCAGCGGCACCGGTGGCGACGTGGAAA AAGGCAAAAAGATCTTCGTGCAGAAATGCGCGCAGTGCCACACGGTGGAAAAAGGCT AAGGTACCCTGACG
C23Cc Primer 1	CGTGCAGCAATTGCACTGAAAAATGCAGGTC
C23Cc Primer 2	CGTCAGGGTACCTTAGCCTTTTTCCACCGTG

Bacterial colony selection procedure/results, and protein expression

Overexpression of the PDZ3-fusions was carried out using the previously reported[3] protocol for cyt *c* expression from the pBTR(hCc) vector except that the bacterial colonies chosen for expression were selected using the protocol discussed below and outlined in Figure S3. All incubation/bacterial growth steps were carried out at 37 °C and all growth media used were supplemented with 50-μg/mL ampicillin unless stated otherwise. The selection procedure was used to select optimally expressing colonies for each PDZ-fusion separately. The expression vectors were individually transformed into BL21* (DE3) *E. coli* (Invitrogen) and grown for ~15 hours on a Luria-Bertani (LB) medium plate. 10 separate 14-mL falcon tubes containing 3-mLs liquid LB were inoculated with 1 colony each originating from the same transformation (all 10 colonies are identical clones) and grown with shaking (160-170 rpm) for exactly 6 hours. After the growth period, 90 μL of each LB pre-growth was transferred individually to 10 14-mL Falcon tubes containing 3 mL of terrific broth (TB) and grown with shaking (160-170 rpm) for ~15-20 hours for protein overexpression. The LB pre-growths used for inoculation were stored at 4 °C until further use. The contents of the TB growths were micro-centrifuged after overexpression and the color of the bacterial pellets was used to determine which colonies of those originally picked from the plates had overexpressed heme protein. Bacterial pellets dark red/brown in color were indicative of heme protein overexpression and those with a light brown color indicated little to no expression. A frozen glycerol stock was prepared from the LB pre-growth corresponding to the chosen colony with visible heme protein overexpression and stored at -80 °C. Overexpression at the 1-L scale was followed as previously reported[5] except that the LB pre-growths were initiated by direct inoculation from glycerol stocks of selected colonies rather than from single random colonies chosen from LB-plates.

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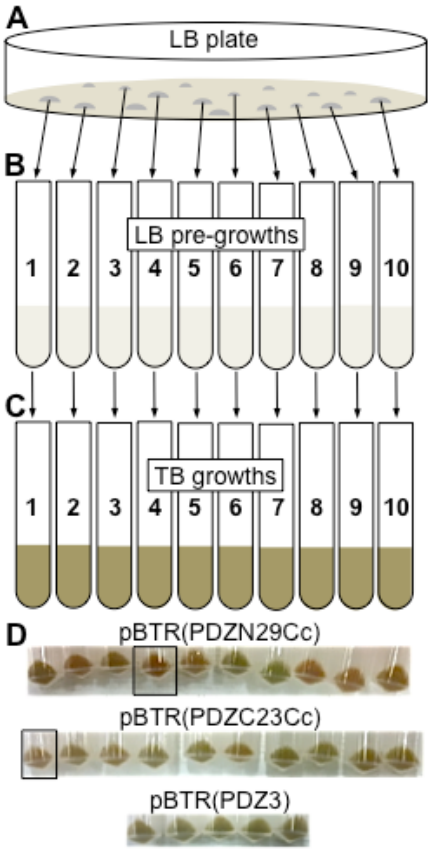

Figure S3. Schematic of the procedure used to select bacterial colonies for overexpression of each PDZ-fusion. (**A**) 10 individual bacterial colonies produced on an LB plate after transformation with a particular vector coding for a PDZ-fusion are used to (**B**) infect 10 LB pre-growths and grown for 6 hours. (**C**) The LB growths are used after expression to start 10 TB growths (richer medium with darker color) for overexpression of the particular PDZ-fusion. (**D**) After the expression period the bacteria from the TB growths are microcentrifuged and PDZ-fusion expression is judged by the pellet color. A sample of the pellets from selected colonies having overexpressed PDZN29Cc (top), PDZC23Cc (middle), and wild-type PDZ3 (bottom) are shown.

Figure S3D shows selected pellets from grows containing overexpressed PDZN29Cc, PDZC23Cc, and pBTR(PDZ3). See main document for discussion. The pellets containing PDZN29Cc (Figure S3D, top) show that at least five colonies show varying levels of overexpression of a heme protein judged by the dark brown/red pellet color. The pellets containing PDZC23Cc (middle) show little to no heme protein overexpression as compared to those containing PDZN29Cc. However, there is variation in the pellet color, with about half having a light brown color, and the other half a green color. The pellets containing wild type PDZ3 all were light brown, with little to no variation in color of the pellets. The cells selected for overexpression on a larger scale is shown in a box, and corresponds to the pellet with the most visibly dark brown/red color compared to the other 9. Because there is no variation in pellet color containing PDZ3, a random pellet was chosen. The selection procedure was reproduced at least three times (30 colonies from the LB plates in total) for each PDZ-fusion to show that the level of expression was the same for each trial. The results of the selection procedure for overexpressed PDZN18Cc were the same as PDZN29Cc. The overexpression of PDZN29Cc in the absence of CCHL (using the pBTR(-CCHL) vector) resulted in pellets with a light brown color, with no variation. The result was the same as that for wild-type PDZ3.

Bacterial lysate clarification and SDS-PAGE gel preparation and staining

The bacterial pellets from a 1-L growth with the same weight for all five samples containing the PDZ-fusions (main document, Fig. 1) were lysed to obtain bacterial extracts/lysates using a previously described procedure.[5] The lysates were adjusted to a pH of 8.8-9.0 using 1-M NaOH and loaded on a Q-Sepharose resin (GE Heatlhcare) pre-equilibrated with 20-mM Tris, pH 9. The column was washed with 10 column volumes of the same buffer and eluted with 20-mM Tris, 500-mM NaCl, pH 9 buffer. 15-mL

of the eluted lysates was concentrated to 1-mL and exchanged in 50-mM sodium phosphate buffer, pH 7.0 (final volume of 2-mL).

12% SDS-PAGE analysis of the partially clarified lysates was performed as previously described.[5] 25 uL of partially clarified and concentrated (see paragraph above for details) lysate for each sample was loaded onto the gel. After electrophoresis, the gels were heme stained, which for denaturing gels reveals only proteins that contain covalently-bound heme, using the procedure described by Braun et. al.[6]

Ultraviolet-visible absorption spectra of lysates

Ultraviolet-visible (UV-vis) absorption spectra of bacterial lysates containing PDZ-N29Cc and PDZ-N18Cc were taken on a Shimadzu UV-2401PC spectrophotometer at room temperature. The spectra were taken immediately after lysis (procedure described above).

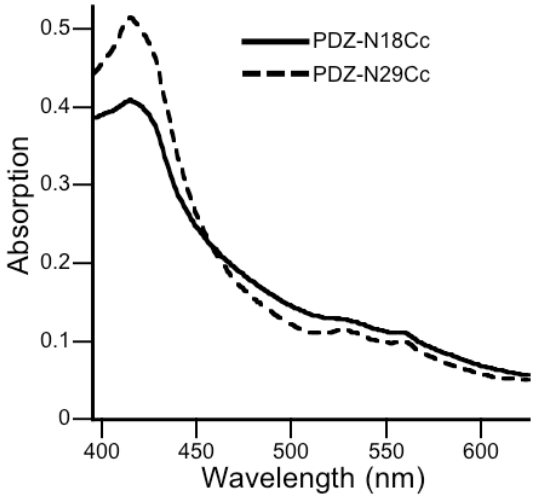

Figure S4. UV-visible absoption spectra of bacterial lysates containing PDZ-N18Cc (solid line) and PDZ-N29Cc (dashed line) taken immediately after the lysis procedure. Both spectra show the signature Soret- and Q-absorption bands for heme containing proteins centered approximately at 414- and 550-nm respectively.

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