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Supplementary Information for

Analysis of allosteric communication in a multienzyme complex by ancestral sequence reconstruction

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This PDF file includes:

Figures S1 to S5
Tables S1 to S7
Legend for Dataset S1
SI References

Other supplementary materials for this manuscript include the following:

Dataset S1

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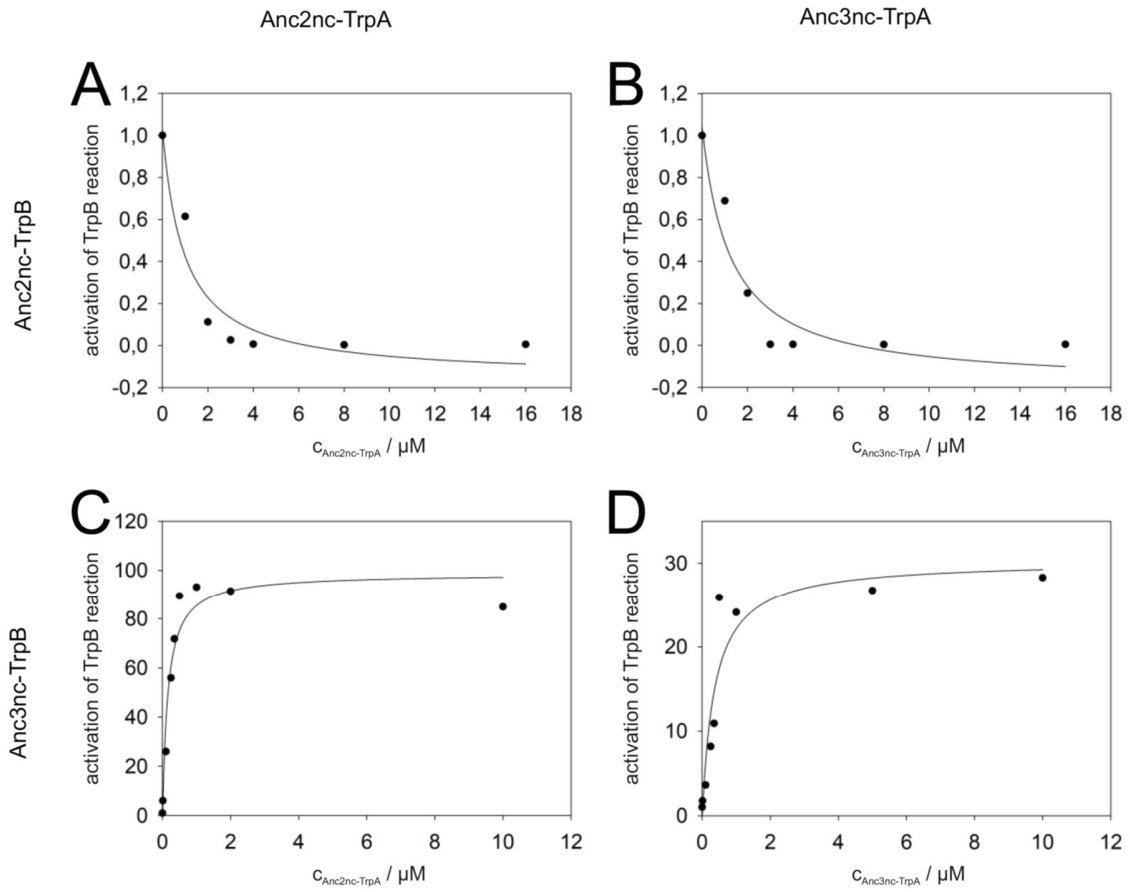

Figure S1. Activity titrations of TrpB subunits with TrpA subunits of Anc2nc-TS and Anc3nc-TS. Anc2nc-TrpB (2 µM, monomer concentration) was titrated with varying concentrations of Anc2nc-TrpA (A) and Anc3nc-TrpA (B). Anc3nc-TrpB (0.5 µM, monomer concentration) was titrated with varying concentrations of Anc2nc-TrpA (C) and Anc3nc-TrpA (D). The titrations were performed at 30 °C in presence of 100 mM KP pH 7.5, 180 mM KCl, 40 µM PLP, 200 µM indole, and 50 mM L-serine for Anc2nc-TrpB or 250 mM L-serine for Anc3nc-TrpB. TrpB activities were followed by monitoring the condensation of indole and L-serine to L-tryptophan at a wavelength of 290 nm.

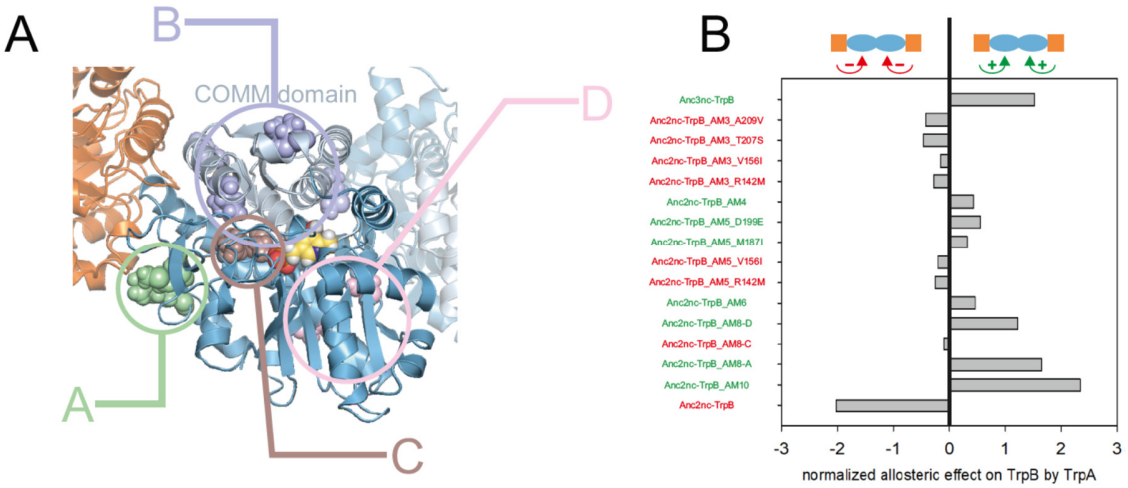

Figure S2. Structural clustering of 10 *a-det* positions in TrpB and subsequent identification of four *a-ess* positions. (**A**) Homology model of Anc2nc-TS with TrpB colored in blue and TrpA colored in orange. The 10 *a-det* positions are represented as spheres, colored according to the clusters they belong to. Cluster A (colored in light green) is located next to the TrpA-TrpB interface. Cluster B (colored in light blue) is positioned within the COMM domain. Cluster C (colored in brown) is located close to the active site, which is indicated by the spherical representation of the PLP cofactor (colored in yellow). Cluster D (colored in pink) lies close to the TrpB-TrpB homodimer interface. (**B**) Assay for the allosteric effect of TrpA on TrpB. All protein variants that contributed to the identification of the four relevant positions *a-ess* are shown. The normalized allosteric effect is defined as the decadic logarithm of the TrpB activity in presence of TrpA divided by the TrpB activity in absence of TrpA. Raw data are given in **Table S6**.

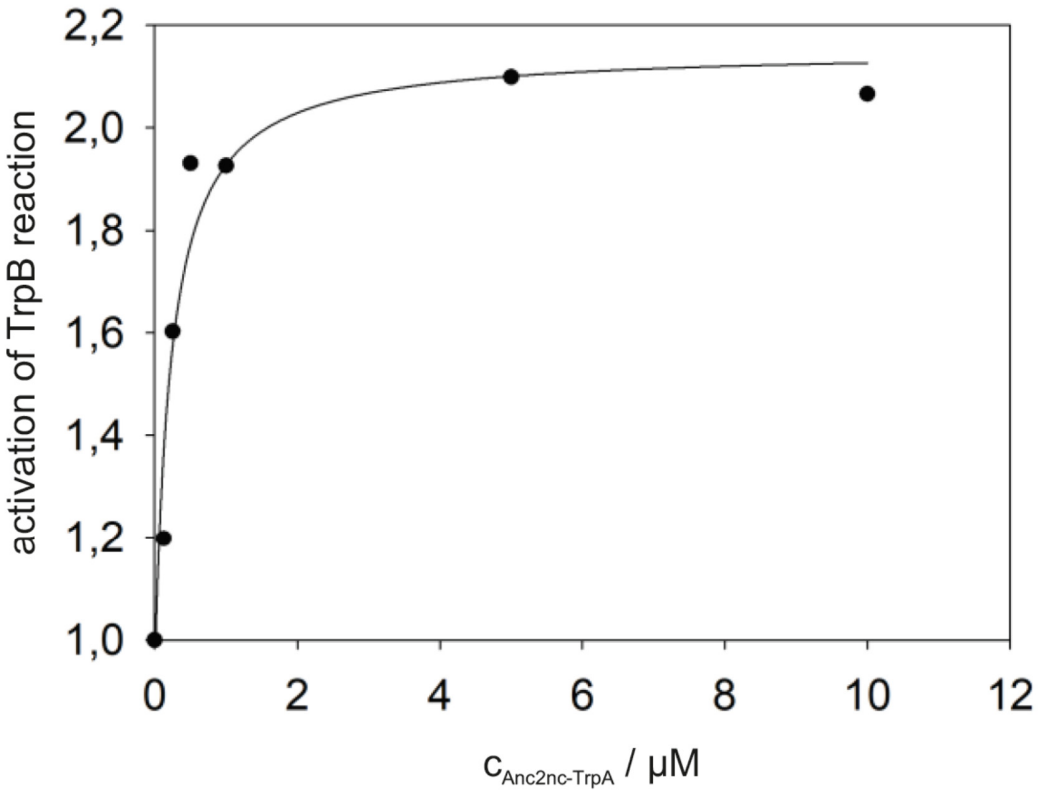

Figure S3. Activity titration of Anc2nc-TrpB_AM4 with Anc2nc-TrpA. TrpB_AM4 (0.5 µM, monomer concentration) was titrated with varying concentrations of Anc2nc-TrpA. The experiment was performed at 30 °C in presence of 100 mM KP pH 7.5, 180 mM KCl, 40 µM PLP, 200 µM indole, 50 mM L-serine. TrpB activity was followed by monitoring the condensation of indole and L-serine to L-tryptophan at a wavelength of 290 nm.

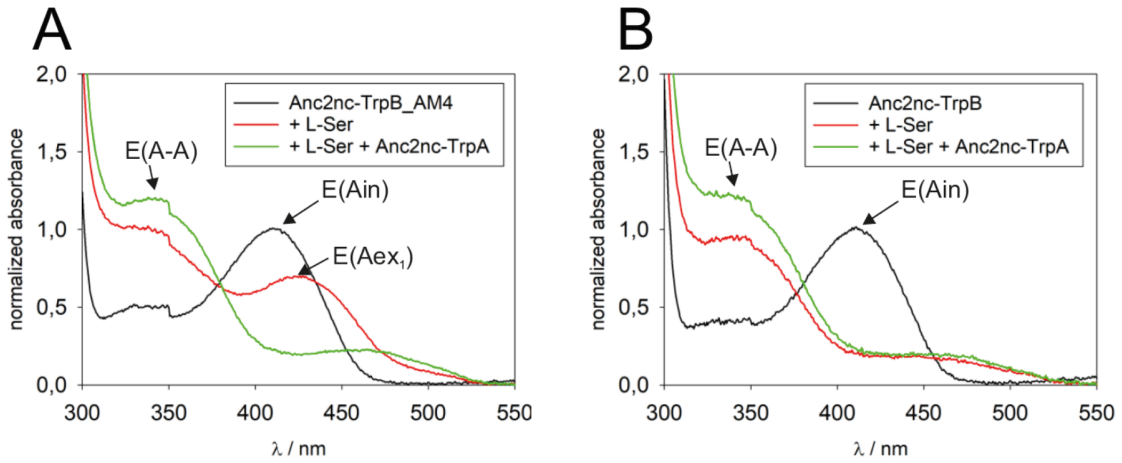

Figure S4. UV/Vis spectra of Anc2nc-TrpB_AM4 and Anc2nc-TrpB in their holo-forms, in presence of L-serine, and in presence of L-serine and Anc2nc-TrpA. (**A**) Anc2nc-TrpB_AM4 (10 µM, monomer concentration) (black), after addition of 20 mM L-serine (red), and after addition of 20 mM L-serine plus 10 µM Anc2nc-TrpA (green). (**B**) Anc2nc-TrpB (10 µM, monomer concentration) (black), after addition of 20 mM L-serine (red), and after addition of 20 mM L-serine plus 10 µM Anc2nc-TrpA (green). Spectra were collected at 30 °C in 50 mM potassium phosphate buffer, pH 7.5. The intermediates of the TrpB reaction (internal aldimine, E(Ain); external aldimine E(Aex1); aminoacrylate, E(A-A)) were identified according to their respective absorbance maxima (1). The spectra were normalized with the value of the absorbance of the E(Ain) peak at 412 nm set to 1.

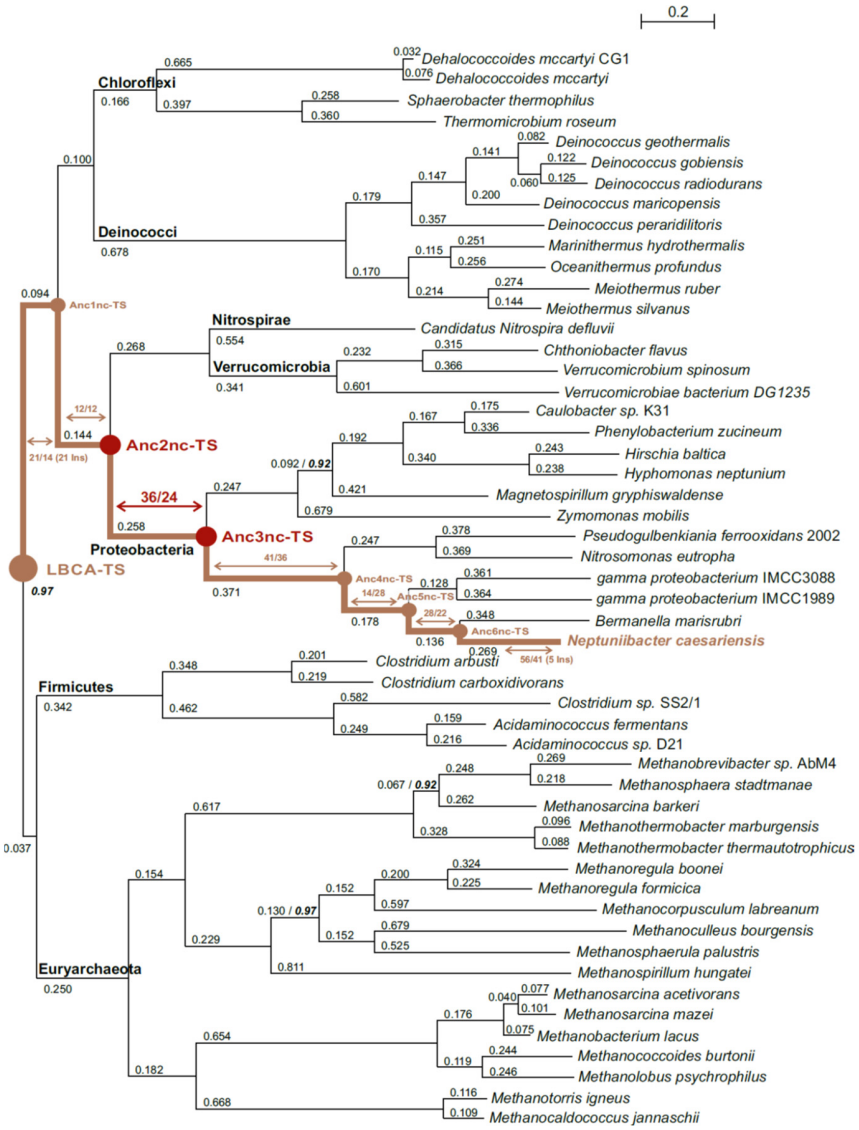

Figure S5. Phylogenetic tree for the reconstruction of ancestral TS. The tree has been midpoint-rooted according to the phylogenetic relationship of extant TS (2). The location of LBCA-TS corresponds to the root and is indicated by a brown dot. The evolutionary path from LBCA-TS to the extant TS of *Neptuniibacter caesariensis* is highlighted in brown and the positions of all intermediate sequences Anc*nc-TS are indicated. The two intermediates (Anc2nc-TS and Anc3nc-TS) that effect the switching from TrpB-inhibition to TrpB-activation by TrpA are printed in red. Next to the double arrows, the number of mutations distinguishing the TrpA/TrpB sequences related to the adjacent nodes are given as well as the number of insertions (Ins) in the TrpB sequences. For all edges, the corresponding rate of mutations per site is listed (compare to the bar at the top, which corresponds to 0.2 mutations per site). To indicate the robustness of the tree, posterior probabilities of all bifurcations are listed. 97 of the posterior probabilities are ≥ 0.99; the four smaller ones, all of which are ≥ 0.92, are printed as numbers formatted in italics and bold.

Table S1. Primer used for site-directed mutagenesis.

Bases in **bold** and underlined indicate mutated positions.

Target construct	Template	Introduced mutation	forward/ reverse	Primer sequence 5'→3'	Method
pET21a_Anc2nc-TrpB_AM10_H24R	pET21a_Anc2nc-TrpB_AM10	H24R	forward reverse	**CGT**TTTGGTCCGTATGGT ACCACGTGCATCCGG	PNK
pET21a_ Anc2nc-TrpB_AM8-A	pET21a_ Anc2nc-TrpB_AM10_H24R	R300M	forward reverse	**ATG**ACCTATCTGCTGC GCTACCATGCAGAAC	PNK
pET21a_ Anc2nc-TrpB_AM10_T207S	pET21a_ Anc2nc-TrpB_AM10	T207S	forward reverse	ACATTATTGGC**TCT**GTTGCCGG CCGGCAAC**AGA**GCCAATAATGT	QCM
pET21a_ Anc2nc-TrpB_AM8-C	pET21a_ Anc2nc-TrpB_AM10_T207S	A209V	forward reverse	TGGCTCTGTT**GTT**GGTCCGCAT ATGCGGACC**AAC**AACAGAGCCA	QCM
pET21a_ Anc2nc-TrpB_AM10_ S73T	pET21a_ Anc2nc-TrpB_AM10	S73T	forward reverse	GTCGTCCG**ACC**CCGCTGTATT AATACAGCGG**GGT**CGGACGAC	QCM
pET21a_ Anc2nc-TrpB_AM8-D	pET21a_ Anc2nc-TrpB_AM10_ S73T	R227E	forward reverse	GCGTTATTGGT**GAA**GAAGCACG TC GACGTGCTTC**TTC**ACCAATAAC GC	QCM
pET21a_ Anc2nc-TrpB_AM7-A_ S73T	pET21a_ Anc2nc-TrpB_AM8-A	S73T	forward reverse	GTCGTCCG**ACC**CCGCTGTATT AATACAGCGG**GGT**CGGACGAC	QCM
pET21a_ Anc2nc-TrpB_AM6	pET21a_ Anc2nc-TrpB_AM7-A_ S73T	R227E	forward reverse	GCGTTATTGGT**GAA**GAAGCACG TC GACGTGCTTC**TTC**ACCAATAAC GC	QCM
pET21a_ Anc2nc-TrpB_AM5_R142M	pET21a_ Anc2nc-TrpB_AM6	R142M	forward reverse	CGTTGCAGCA**ATG**TTTGGTCTG GAAT ATTCCAGACCAAA**CAT**TGCTGC AACG	QCM
pET21a_ Anc2nc-TrpB_AM5_V156I	pET21a_ Anc2nc-TrpB_AM6	V156I	forward reverse	GTGCCGAAG**ATA**TTGAACGTCA G CTGACGTTCAA**TAT**CTTCGGCA C	QCM
pET21a_ Anc2nc-TrpB_AM5_M187I	pET21a_ Anc2nc-TrpB_AM6	M187I	forward reverse	TGAAAGATGCA**ATT**AATGAAGC AATG CATTGCTTCATT**AAT**TGCATCTT TCA	QCM
pET21a_ Anc2nc-TrpB_AM5_D199E	pET21a_ Anc2nc-TrpB_AM6	D199E	forward reverse	TACCAATGTG**GAA**GATACCTAT TAC GTAATAGGTATC**TTC**CACATTGG TA	QCM
pET21a_ Anc2nc-TrpB_AM4	pET21a_ Anc2nc-TrpB_AM5_M187I	D199E	forward reverse	TACCAATGTG**GAA**GATACCTAT TAC GTAATAGGTATC**TTC**CACATTGG TA	QCM

Table S2. Amino acid sequences of experimentally characterized proteins. Sequence colored in red: His_6-tag for IMAC purification. In case of pMAL-c5T constructs, the His_6-MBP-tag sequence contains a thrombin cleavage site (LVPR|GS) which was exploited to remove the His_6-MBP-tag from the desired amino acid sequence. The residual GSH sequence is colored in green. Sequence colored in blue: Initial methionine (M1). Sequence in **bold** and underlined: Residues that were mutated compared to the respective wild-type sequence.

Construct	Protein	Studied amino acid sequence
pET21a_LBCA-TrpA (2)	LBCA-TrpA	MNRIAEAFEELKKKGEKALIPFITAGDPDLETTLELVRALVEAGADIIELGIPFSDPLADGPTIQRASQR ALASGTTLDKVFEMVRELREKNTDVPIVFLTYYNPIFRYGIERFVKECAEAGVDGLIVPDLPPEEAADL AAAAEKYGVDLIFLVAPTSTDERIKMIAKHASGFVYCVSVTGVTGARSEIAADLAELVSRIRKHTDLPI AVGFGISTPEQAAEVAQVADGVIVGSAIVKRIEENQDEEDIVEEVREFVRELREAVKLEHHHHHH
pET21a_LBCA-TrpB (2)	LBCA-TrpB	MIGRFGKYGGQYVPETLMPALEELEEAYERAKNDPEFQAELEYYLRDYVGRPTPLYFAENLTKDLG GAKIYLKREDLNHTGAHKINNALGQALLAKRMGKKRVIAETGAGQHGVATATVAAMFGLECVVY MGAEDIERQALNVFRMKLLGAKVRPVTSGSRTLKDAINEAMRDWVTNVEDTFYIIGSVVGPHPYP MMVRDFQSVIGEEARQQILEKEGRLPDAIVACVGGGSNAMGIFHPFIDDESVRLIGVEAAGKGIET GKHAATLSAGRPGVLHGAMTYLLQDEDGQIIEAHSISAGLDYPGVGPEHAYLKDTGRAEYVSVTDD EALEAFQLLSRTEGIIPALESSHAVAYAMKLAPELSKDQIIVVNLSGRGDKDVNTVARYLLGVELDLE HHHHHH
pUR28a_Anc1nc-TrpA	Anc1nc-TrpA	MHHHHHHLEMNRIAEAFAKLKAEGEKALIPYITAGDPDLETTLELVRALVEAGADIIELGIPFSDPLA DGPTIQRASQRALASGTTLAKVLEMVRELREKNTDVPIVFMTYYNPIYSYGLERFVKECAEAGVDGL IVPDLPPEEAADLAAAARKHGLDLIFLLAPTSTDERIKLIAKHASGFVYCVSVTGVTGARSELAADLAE LVSRIRKHTDLPIAVGFGISTPEQAAEVAQVADGVVVGSAIVKRIEENQDEEDLVEEVAAFVRELREA VK
pET21a_Anc1nc-TrpB	Anc1nc-TrpB	MNVSTNVMATTTKSKAALPDARGRFGKYGGRYVPETLMPALEELEEAYERAKRDPEFQAELEYYLK DYVGRPTPLYFAENLTEHLGGAKIYLKREDLNHTGAHKINNALGQALLAKRMGKKRVIAETGAGQH GVATATVAAMFGLECVVYMGAEDIERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDWVT NVEDTYYIIGSVVGPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDAIVACVGGGSNAMGIFHPFI DDESVRLIGVEAAGEGIETGKHAASLSAGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPGVGPE HSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSGRGDK DVNTVARYLLGVELDLEHHHHHH
pUR28a_Anc2nc-TrpA	Anc2nc-TrpA	MHHHHHHLEMNRIAEAFAKLKAEGKKALIPYITAGDPDLETTLELVRALVEAGADIIELGVPFSDPLA DGPVIQRASQRALASGTTLRKVLEMVRELREKNTEVPIVLMTYYNPIYSYGLERFVKEAAEAGVDGLI VPDLPPEEAADLAAAAREHGLDLIFLLAPTSTDERIKLIAKHASGFVYYVSVTGVTGARSELAADLAEK VARIRKHTDLPIAVGFGISTPEQAAEVAQVADGVVVGSAIVKRIEENQDEEDLVAEVAAFVRELREA VK
pET21a_Anc2nc-TrpB	Anc2nc-TrpB	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAAMFGLECVVYMGAEDIERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNVEDTYYIIGSVVGPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSG RGDKDVNTVARYLLGVELDLEHHHHHH
pUR28a_Anc3nc-TrpA	Anc3nc-TrpA	MHHHHHHLEMNRIAAAFAKLKAEGKKALIPYITAGDPDLETTLELMHALVEAGADIIELGVPFSDP MADGPVIQRASQRALASGTTLRDVLEMVAEFRETDTETPIVLMGYANPIYSMGYERFAKAAAEAG VDGLIVVDLPPEEAAELAAALREHGIDLIFLLAPTSPDERIKLIAEHASGFVYYVSVTGVTGARSADAA DVAAKVARIRKHTDLPIAVGFGIKTPEQAAEVAKVADGVVVGSAIVNEIEEAQDEENLTAAVAALVR ELRAAVK
pET21a_Anc3nc-TrpB	Anc3nc-TrpB	MNASTNVSATTPKSYAALPDARGHFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYLK HYVGRPSPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQH GVATATVAARFGLECVVYMGAEDVERQALNVFRMKLLGAEVRPVTSGSRTLKDAMNEAMRDW VTNVDDTFYIIGTVAGPHPYPMMVRDFQSVIGREARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGNRTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSWLKDIGRAEYVSVTDDEALAAFQLLSRLEGIIPALESSHALAYAAKLAPTMSKDQIIVVNLS GRGDKDVNTVARYLLGVDLDLEHHHHHH
pUR28a_Anc4nc-TrpA	Anc4nc-TrpA	MHHHHHHLEMNRIAACFAKLKAEGKKALIPYITAGDPDPDTTVDLMHALVEAGADIIELGVPFSDP MADGPVIQRASERALAHNTSLRDVLEMVAEFRETDTETPVVLMGYANPIEAMGYERFAKAAAEA GVDGVLTVDLPPEEAAELAAALKEHGIDPIFLLAPTTPEQRVKLIAEHASGYVYYVSVKGVTGAGNL DVDDVAAKLARIRQHTDLPIGVGFGIKDGETAASVAEVADGVVVGSALVNKIEEAQDEDNLKAAV AALVAELRAAVD
pET21a_Anc4nc-TrpB	Anc4nc-TrpB	MNASTNVSAISLKSYAQLPDARGHFGPYGGRFVAETLMAALDELEEAYERAKRDPAFQAEFDRDL KHYVGRPSPLYFAERWTEHLGGAKIYLKREDLNHTGAHKVNNTIGQALLAKRMGKKRVIAETGAG QHGVASATVAARFGLECVVYMGAEDVERQALNVYRMKLLGAEVVPVTSGSRTLKDAMNEAMRD WVTNVDDTFYIIGTVAGPHPYPMMVRDFQSVIGREARQQMLEQEGRLPDALVACVGGGSNAIGL FHPFIEDEGVRMIGVEAGGHGIETGKHAAPLSAGRPGVLHGNRTYLMQDEDGQIIETHSVSAGLD YPGVGPEHSWLKDIGRAEYVAVTDDEALAAFHALTRIEGIMPALESSHALAYAAKLAPTMSKDQIIV VNLSGRGDKDINTVAQYLSGINLDLEHHHHHH

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pET21a_Anc2nc-TrpB_AM8-C	Anc2nc-TrpB_AM8-C	MNVSTNVMATTPKSKAALPDARG**H**FGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRP**S**PLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRD WVTNV**D**DTYYIIGSVVGPHPYPMMVRDFQSVIG**R**EARQQILEKEGRLPDALVACVGGGSNAMGL FHPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGS**R**TYLLQDEDGQIIEAHSISAGLDYP GVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLS GRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM8-D	Anc2nc-TrpB_AM8-D	MNVSTNVMATTPKSKAALPDARG**H**FGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPT PLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRD WVTNV**D**DTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGL FHPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGS**R**TYLLQDEDGQIIEAHSISAGLDYP GVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLS GRGDKDVNTVARYLLGVELDLEHHHHHH

pET21a_Anc2nc-TrpB_AM6	Anc2nc-TrpB_AM6	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRD WVTNV**D**DTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGL FHPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDY PGVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNL SGRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM5_R142M	Anc2nc-TrpB_AM5_R142M	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAAMFGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRD WVTNV**D**DTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGL FHPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDY PGVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNL SGRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM5_V156I	Anc2nc-TrpB_AM5_V156I	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAEDIERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRDW VTNV**D**DTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLF HPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYP GVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLS GRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM5_M187I	Anc2nc-TrpB_AM5_M187I	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNV**D**DTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLF HPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYP GVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLS GRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM5_D199E	Anc2nc-TrpB_AM5_D199E	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDA**M**NEAMRD WVTNVEDTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGL FHPFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDY PGVGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNL SGRGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM4	Anc2nc-TrpB_AM4	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNVEDTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSG RGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM3_ R142M	Anc2nc-TrpB_AM3_R142M	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAAMFGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNVEDTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSG RGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM3_ V156I	Anc2nc-TrpB_AM3_V156I	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAEDIERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDWV TNVEDTYYIIG**T**V**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFHP FIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPGV GPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSGR GDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM3_T207S	Anc2nc-TrpB_AM3_T207S	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNVEDTYYIIGSV**A**GPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSG RGDKDVNTVARYLLGVELDLEHHHHHH
pET21a_Anc2nc-TrpB_AM3_A209V	Anc2nc-TrpB_AM3_A209V	MNVSTNVMATTPKSKAALPDARGRFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYL KEYVGRPTPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQ HGVATATVAA**R**FGLECVVYMGAED**V**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAINEAMRDW VTNVEDTYYIIG**T**VVGPHPYPMMVRDFQSVIGEEARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGSMTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSYLKDTGRAEYVSVTDDEALEAFQLLSRTEGIIPALESSHAIAYAVKLAPEMSKDQIIVVNLSG RGDKDVNTVARYLLGVELDLEHHHHHH

pET21a_LBCA-TrpB_AM4	LBCA-TrpB_AM4	MIGRFGKYGGQYVPETLMPALEELEEAYERAKNDPEFQAELEYYLRDYVGRPTPLYFAENLTKDLG GAKIYLKREDLNHTGAHKINNALGQALLAKRMGKKRVIAETGAGQHGVATATVAA**R**FGLECVVY MGAED**V**ERQALNVFRMKLLGAKVRPVTSGSRTLKDAINEAMRDWVTNVEDTFYIIG**T**V**A**GPHPY PMMVRDFQSVIGEEARQQILEKEGRLPDAIVACVGGGSNAMGIFHPFIDDESVRLIGVEAAGKGIE TGKHAATLSAGRPGVLHGAMTYLLQDEDGQIIEAHSISAGLDYPGVGPEHAYLKDTGRAEYVSVTD DEALEAFQLLSRTEGIIPALESSHAVAYAMKLAPELSKDQIIVVNLSGRGDKDVNTVARYLLGVELDL EHHHHHH
pET21a_Anc3nc TrpB_AM4	Anc3nc-TrpB_AM4	MNASTNVSATTPKSYAALPDARGHFGPYGGRYVPETLMPALEELEEAYERAKRDPAFQAELDYYLK HYVGRPSPLYFAERLTEHLGGAKIYLKREDLNHTGAHKINNAIGQALLAKRMGKKRVIAETGAGQH GVATATVAA**M**FGLECVVYMGAED**I**ERQALNVFRMKLLGAEVRPVTSGSRTLKDAMNEAMRDW VTNVDDTFYIIG**S**V**V**GPHPYPMMVRDFQSVIGREARQQILEKEGRLPDALVACVGGGSNAMGLFH PFIDDEGVRMIGVEAGGHGIETGKHAASLSGGRPGVLHGNRTYLLQDEDGQIIEAHSISAGLDYPG VGPEHSWLKDIGRAEYVSVTDDEALAAFQLLSRLEGIIPALESSHALAYAAKLAPTMSKDQIIVVNLS GRGDKDVNTVARYLLGVDLDLEHHHHHH

Table S3. Strategy for the identification of positions relevant for the direction of the allosteric effect of TrpA on TrpB.

The color code is as in **Figure S2A**: Mutations colored in light green are located in Cluster A. Mutations colored in light blue are located in Cluster B. Mutations colored in brown are located in Cluster C. Mutations colored in pink are located in Cluster D. The activation (+) or inhibition (-) column is based on experimental results shown in **Figure S2B**.

Step	Protein	Mutated positions	Allosteric effect on TrpB	Conclusion
#1 "wildtype" Anc2nc-TrpB	Anc2nc-TrpB	none	−	none
#2 Anc2nc-TrpB with 10 unique positions mutated towards Anc3nc identity	Anc2nc-TrpB_AM10	R24H M300R M142R I156V I187M E199D S207T V209A T73S E227R	+	10 mutated positions are sufficient to inverse the allosteric effect on TrpB
#3 Anc2nc-TrpB_AM10 Cluster A, C and D seperatly backmutated to Anc2nc identity	Anc2nc-TrpB_AM8-A	M142R I156V I187M E199D S207T V209A T73S E227R	+	R24H M300R are not required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM8-C	R24H M300R M142R I156V I187M E199D T73S E227R	−	S207T V209A are required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM8-D	R24H M300R M142R I156V I187M E199D S207T V209A	+	T73S E227R are not required for inversion of the allosteric effect on TrpB
#4 Combinational backmutation of the irrelevant positions from Cluster A and D	Anc2nc-TrpB_AM6	M142R I156V I187M E199D S207T V209A	+	the 6 mutated positions are sufficient to inverse the allosteric effect on TrpB

Step	Protein	Mutated positions	Allosteric effect on TrpB	Conclusion
#5 Anc2nc-TrpB_AM6 single backmutation of the positions in Cluster B	Anc2nc-TrpB_AM5_R142M	I156V I187M E199D S207T V209A	−	M142R is required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM5_V156I	M142R I187M E199D S207T V209A	−	I156V is required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM5_M187I	M142R I156V E199D S207T V209A	+	I187M is not required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM5_D199E	M142R I156V I187M S207T V209A	+	E199D is not required for inversion of the allosteric effect on TrpB
#6 Combinational backmutation of the irrelevant positions identified in #5	Anc2nc-TrpB_AM4	M142R I156V S207T V209A	+	the 4 mutated positions are sufficient to inverse the allosteric effect on TrpB
#7 Anc2nc-TrpB_AM4 single backmutation of the last 4 positions	Anc2nc-TrpB_AM3_R142M	I156V S207T V209A	−	M142R is required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM3_V156I	M142R S207T V209A	−	I156V is required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM3_T207S	M142R I156V V209A	−	S207T is required for inversion of the allosteric effect on TrpB
	Anc2nc-TrpB_AM3_A209V	M142R I156V S207T	−	V209A is required for inversion of the allosteric effect on TrpB

Table S4. Tryptophan formation by TrpB-subunits in absence and presence of the respective TrpA-subunits followed by an HPLC-based assay.

Experimental conditions: 100 mM EPPS/KOH pH 7.5, 180 mM KCl, 40 µM PLP, 45 mM L-serine, 300 µM indole, 0.5 µM TrpB (monomer) and 1 µM TrpA (monomer) at 30 °C. The mean and the standard deviations of two independent measurements are given.

Protein	Initial rate of tryptophan formation / nM/s
LBCA-TrpB	630 ± 110
LBCA-TrpA + LBCA-TrpB	480 ± 140
Anc1nc-TrpB	600 ± 37
Anc1nc-TrpA + Anc1nc-TrpB	360 ± 67
Anc2nc-TrpB	870 ± 170
Anc2nc-TrpA + Anc2nc-TrpB	100 ± 38
Anc3nc-TrpB	62 ± 37
Anc3nc-TrpA + Anc3nc-TrpB	580 ± 89
Anc4nc-TrpB	370 ± 220
Anc4nc-TrpA + Anc4nc-TrpB	860 ± 140
Anc5nc-TrpB	450 ± 75
Anc5nc-TrpA + Anc5nc-TrpB	850 ± 87
Anc6nc-TrpB	240 ± 67
Anc6nc-TrpA + Anc6nc-TrpBB	550 ± 95
ncTrpB	41 ± 33
ncTrpA + ncTrpB	350 ± 43

Table S5. Tryptophan formation of TrpB-subunits in absence and presence of the respective TrpA-subunits followed by absorbance spectroscopy at 290 nm.

Experimental conditions: 100 mM potassium phosphate pH 7.5, 180 mM KCl, 40 µM PLP, 50 mM L-serine, 200 µM indole, 0.5 µM TrpB (monomer) and 10 µM TrpA (monomer) at 30 °C. The mean and the standard deviations of two independent measurements are given.

Protein	Initial rate of tryptophan formation / nM/s
Anc2nc-TrpB	150 ± 0.4
Anc2nc-TrpB + Anc2nc-TrpA	1.5 ± 0.0
Anc2nc-TrpB_AM10	0.6 ± 2.0
Anc2nc-TrpB_AM10 + Anc2nc-TrpA	120 ± 4.4
Anc3nc-TrpB	14 ± 0.7
Anc3nc-TrpB + Anc3nc-TrpA	480 ± 25
Anc3nc-TrpB_AM10	600 ± 31
Anc3nc-TrpB_AM10 + Anc3nc-TrpA	9.6 ± 1.2

Table S6. Tryptophan formation of TrpB-subunits in absence and presence of the respective TrpA-subunits followed by absorbance spectroscopy at 290 nm.

Experimental conditions: 100 mM potassium phosphate pH 7.5, 180 mM KCl, 40 µM PLP, 50 mM L-serine, 200 µM indole, 0.5 µM TrpB (monomer) and 10 µM TrpA (monomer) at 30 °C. The mean and the standard deviations of two independent measurements are given.

Protein	Initial velocity of tryptophan formation / nM/s
Anc2nc-TrpB	150 ± 0.4
Anc2nc-TrpB + Anc2nc-TrpA	1.5 ± 0.0
Anc2nc-TrpB_AM10	0.6 ± 2.0
Anc2nc-TrpB_AM10 + Anc2nc-TrpA	120 ± 4.4
Anc2nc-TrpB_AM8-A	6.3 ± 1.4
Anc2nc-TrpB_AM8-A + Anc2nc-TrpA	280 ± 12
Anc2nc-TrpB_AM8-C	5.2 ± 0.5
Anc2nc-TrpB_AM8-C + Anc2nc-TrpA	4.2 ± 0.1
Anc2nc-TrpB_AM8-D	18 ± 2.8
Anc2nc-TrpB_AM8-D + Anc2nc-TrpA	300 ± 20
Anc2nc-TrpB_AM6	110 ± 0.2
Anc2nc-TrpB_AM6 + Anc2nc-TrpA	340 ± 38
Anc2nc-TrpB_AM5_R142M	360 ± 19
Anc2nc-TrpB_AM5_R142M + Anc2nc-TrpA	200 ± 7.9
Anc2nc-TrpB_AM5_V156I	290 ± 3.6
Anc2nc-TrpB_AM5_V156I + Anc2nc-TrpA	180 ± 9.4
Anc2nc-TrpB_AM5_M187I	105 ± 4.2
Anc2nc-TrpB_AM5_M187I + Anc2nc-TrpA	220 ± 1.9
Anc2nc-TrpB_AM5_D199E	51 ± 2.3
Anc2nc-TrpB_AM5_D199E + Anc2nc-TrpA	190 ± 3.8
Anc2nc-TrpB_AM4	100 ± 1.2

Anc2nc-TrpB_AM4 + Anc2nc-TrpA	270 ± 2.3
Anc2nc-TrpB_AM3_R142M	320 ± 32
Anc2nc-TrpB_AM3_R142M + Anc2nc-TrpA	170 ± 8.5
Anc2nc-TrpB_AM3_V156I	190 ± 6.1
Anc2nc-TrpB_AM3_V156I + Anc2nc-TrpA	130 ± 7.0
Anc2nc-TrpB_AM3_T207S	180 ± 0.2
Anc2nc-TrpB_AM3_T207S + Anc2nc-TrpA	62 ± 0.1
Anc2nc-TrpB_AM3_A209V	91 ± 0.8
Anc2nc-TrpB_AM3_A209V + Anc2nc-TrpA	35 ± 1.1

Table S7. Tryptophan formation of TrpB-subunits in absence and presence of the respective TrpA-subunits followed by absorbance spectroscopy at 290 nm.

Experimental conditions: 100 mM potassium phosphate pH 7.5, 180 mM KCl, 40 µM PLP, 50 mM L-serine, 200 µM indole, 0.5 µM TrpB (monomer) and 10 µM TrpA (monomer) at 30 °C. The mean and the standard deviations of two independent measurements are given.

Protein	Initial rate of tryptophan formation / nM/s
Anc3nc-TrpB	7.1 ± 0.2
Anc3nc-TrpB + Anc3nc-TrpA	420 ± 7.7
Anc3nc-TrpB_AM4	120 ± 5.6
Anc3nc-TrpB_AM4 + Anc3nc-TrpA	25 ± 4.4
LBCA-TrpB	290 ± 16
LBCA-TrpB + LBCA-TrpA	39 ± 3.3
LBCA-TrpB_AM4	36 ± 2.6
LBCA-TrpB_AM4 + LBCA-TrpA	170 ± 1.6

Dataset S1 (separate file). Posterior probabilities for all residues of LBCA-TS, Anc1nc-TS - Anc6nc-TS in Excel format. The posterior probabilities are the outcome of the ASR by means of FastML (3) and given for all residues of the reconstructed TrpA and TrpB sequences.

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