Assessment of interkingdom TIR domain interactions using a modular yeast tripartite split-GFP system

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Supplementary material

Contents:

A

Anti-6xHis

48 —
35 —
25 —

β10-BtpA | BtpA-β10 | β10-BtpA(TIR) | BtpA(TIR)-β10 | β10-BtpB | BtpB-β10 | β10-BtpB(TIR) | BtpB(TIR)-β10 | Empty vector

◄ Btp

*

◄ Btp(TIR)

Anti-G6PDH

B

Anti-FLAG

48 —
35 —
25 —

β11-BtpA | BtpA-β11 | β11-BtpA(TIR) | BtpA(TIR)-β11 | β11-BtpB | BtpB-β11 | β11-BtpB(TIR) | BtpB(TIR)-β11 | Empty vector

◄ Btp

◄ Btp(TIR)

Anti-G6PDH

C

Anti-6xHis

135 —
100 —
63 —
48 —
35 —

β11-MyD88 | MyD88-β11 | β11-TRAM | TRAM-β11 | β11-TIRAP | TIRAP-β11 | β11-TRIF | TRIF-β11 | Empty vector

*

Anti-G6PDH

Supplementary Figure S1. Immunoblots showing expression of the expressed protein fusions in this work. A. Expression of GFP-β10 fusions of BtpA and BtpB or their TIR domais alone (as indicated) fused in their N- or C-termini to GFP-β10 as indicated. Fusions were immunodetected with anti-polyHis antibodies, as a 3×FLAG-6×His tag was fused at the opposite side of the GFP-β10 sheet. **B.** Expression of GFP-β11 fusions of BtpA and BtpB or their TIR domais alone fused in their N- or C-termini to GFP-β11, as indicated. Fusions were immunodetected with anti-FLAG antibodies. **C.** Expression of human TIR-containing proteins fused in their N- or C-termini to GFP-β11, as indicated. In all cases, cells were grown for 4 h in galactose-based medium before collection for lysate preparation. Non-specific bands are marked with asterisks (*). Anti-G6PDH antibody was used in all cases as a loading control. Representative cropped immunoblots are shown. The corresponding original uncropped and non-contrasted blots are shown in Supplementary Figure S6.

A

β11-BtpA^TIR   β10-BtpA   BtpA-β10

BtpA^TIR-β11

B

β11-BtpB^TIR   β10-BtpB   BtpB-β10

BtpB^TIR-β11

C

β11-BtpB   β10-BtpA   BtpA-β10

BtpB-β11

D

β11-BtpB^TIR   β10-BtpA^TIR   BtpA^TIR-β10

BtpB^TIR-β11

E

β11-BtpB^TIR   β10-BtpA   BtpA-β10

BtpB^TIR-β11

F

β11-BtpB   β10-BtpA^TIR   BtpA^TIR-β10

BtpB-β11

G

FIM fold

2xNt
Nt+Ct
Ct+Nt
2xCt

BtpA+BtpATIR   BtpB+BtpBTIR   BtpA+BtpB   BtpATIR+BtpBTIR   BtpA+BtpBTIR   BtpATIR+BtpB
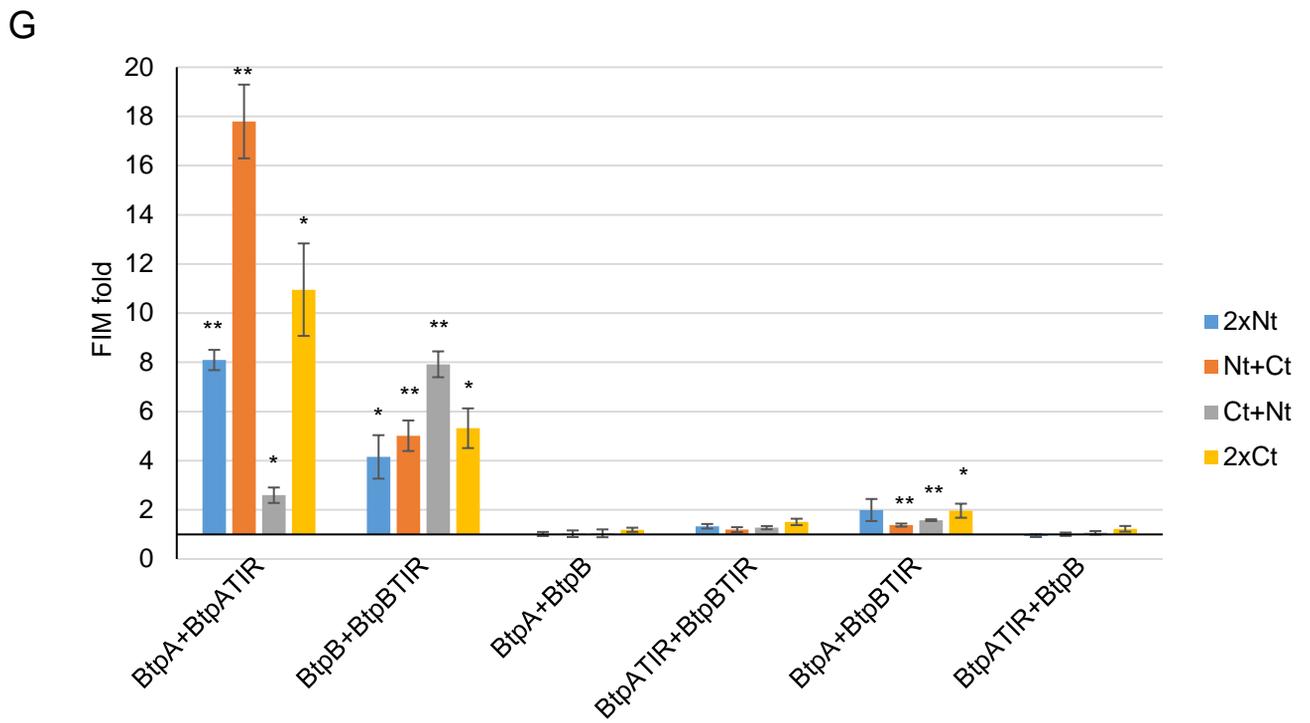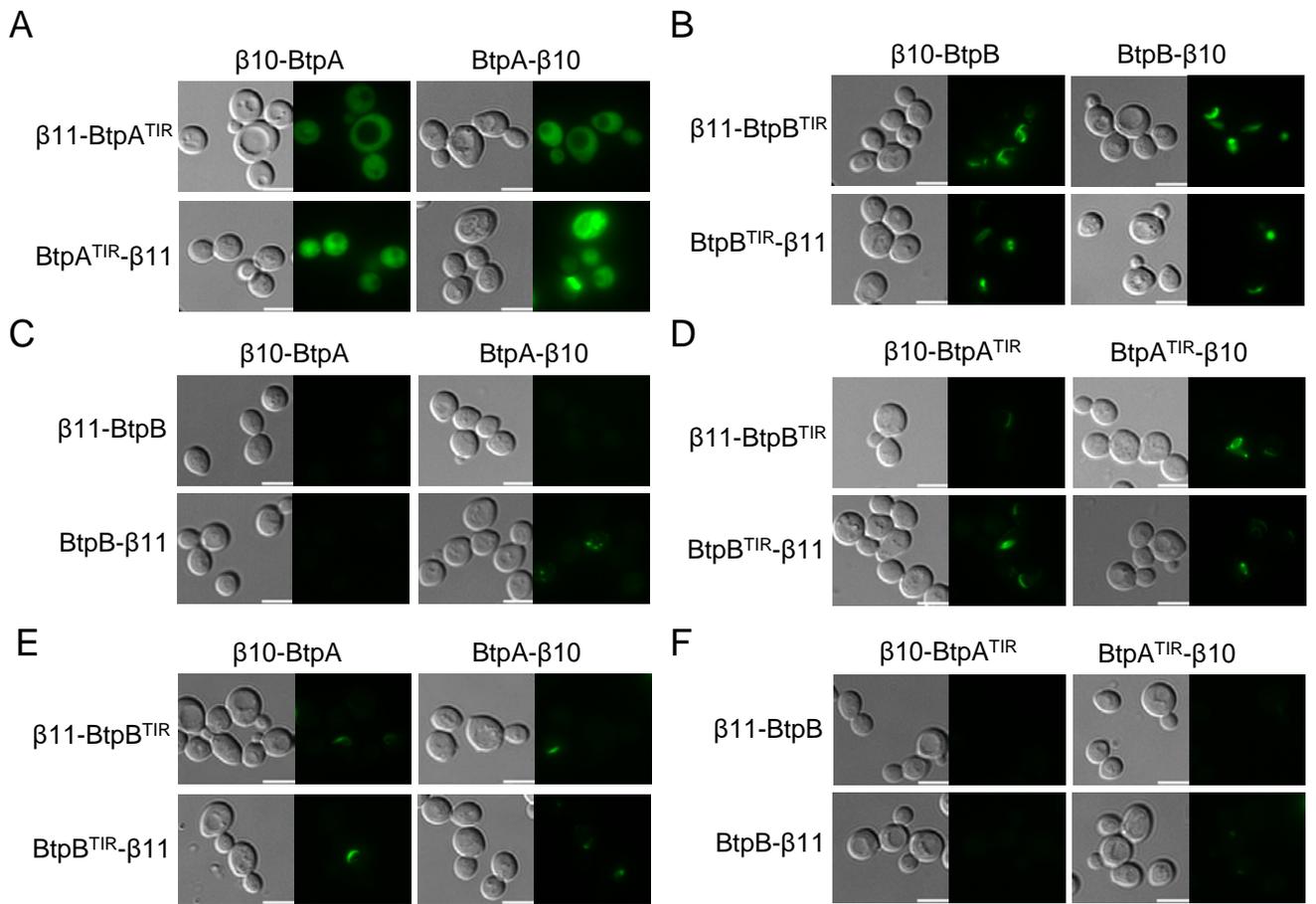
Supplementary Figure S2. Microscopy and flow cytometry data from heterotypic BtpA and BtpB full-length and TIR-only combinations. A-F. Representative bright field and green fluorescence images of all combinations of interactions for BtpA-BtpA(TIR) (**A**), BtpB-BtpB (**B**), BtpA-BtpB (**C**), BtpA(TIR)-BtpB(TIR) (**D**), BtpA-BtpB(TIR) (**E**), and BtpA(TIR)-BtpB (**F**). As indicated, in each panel, 2×Nt, Nt-Ct, Ct-Nt, and 2×Ct combinations are represented in the first, second, third and fourth quarters, respectively. In all cases the point mutations BtpA^{E217A} or BtpB^{E234A} were present to elude TIR toxicity in yeast. AF1 yeast strain was co-transformed with the combination of GFP β10 and β11 strand plasmids, as indicated, and incubated for 4 h in SG galactose-based medium before microscopic visualization. Bars represent 5 μm. **B.** Fluorescence intensity mean normalized to that of the negative control bearing the empty vectors (FIM fold) as derived from flow cytometry histograms of the same combinations as in (A). Data are the average of three experimental replicates on three different transformant clones. Error bars represent the standard deviation (SD). Asterisks indicate a p value <0.05 (*), <0.01 (**), or <0.001 (***) calculated using Student's t test.

A BtpA dimer

B BtpA hexamer

C BtpB dimer

D BtpB hexamer

E

	ipTM	
	BtpA	**BtpB**
2x	0.57	0.2
3x	0.19	0.33
4x	0.19	0.47
5x	0.18	0.49
6x	0.17	0.52

Scored Residue

Expected Position Error (Ångströms)
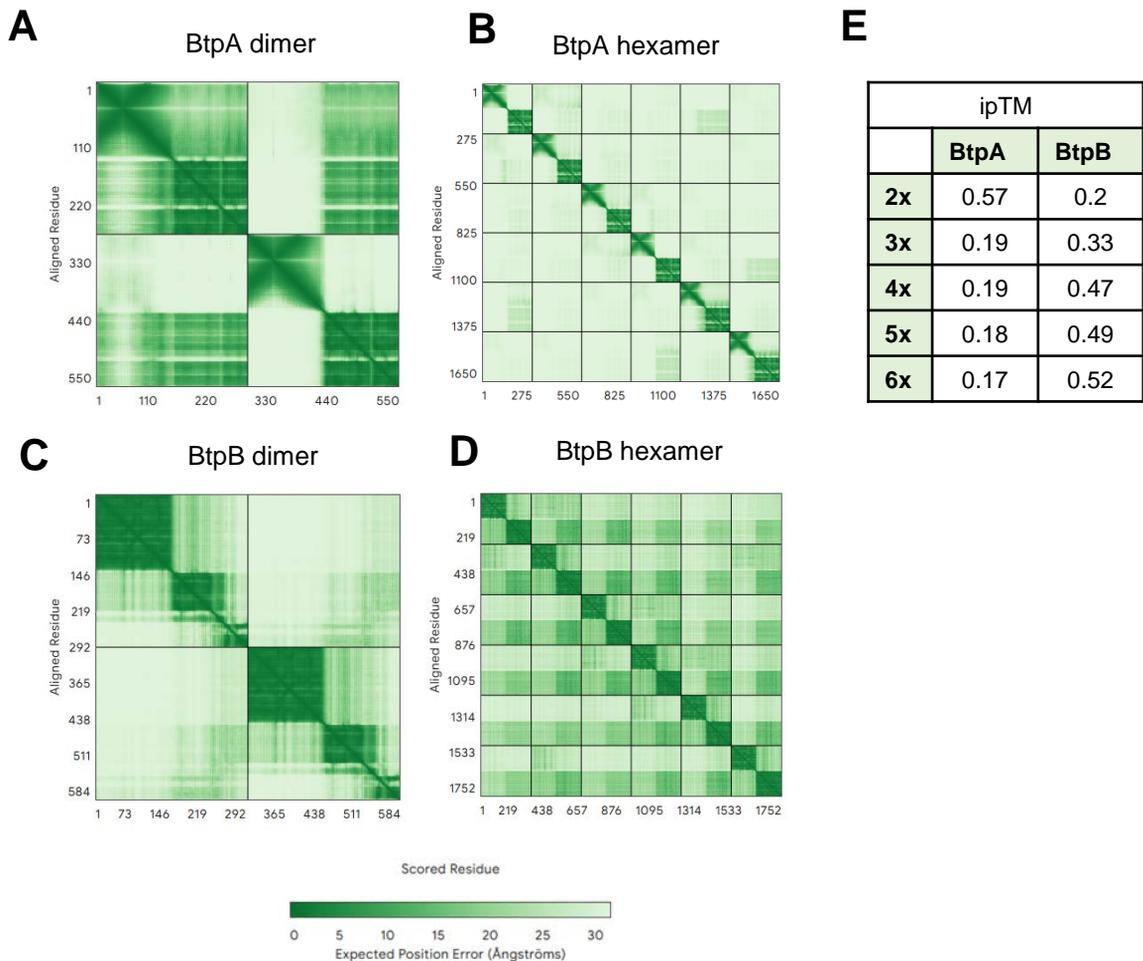
Supplementary Figure S3. AlphaFold multimer predictions for BtpA and BtpB homopolymers. AlphaFold3 PAE (Predicted Aligned Error) plots for structural models of a BtpA dimer (**A**), BtpA hexamer (**B**), BtpB dimer (**C**), and BtpB hexamer (**D**). The sequences of two to six molecules of each protein were plotted along both axes in sequential order. As shown in (**E**), predictions were less robust with the addition of further monomers for BtpA, whereas in the case of BtpB predictions were more robust with the addition of further monomers. The **ipTM** (Interface Predicted Template Modeling score) parameter is a proxy to the likeliness of interaction. The PAE plots in (**A**) and (**D**) correspond to the models shown in Figure 3A. Diagonal quadrants display the predicted alignment error within the individual molecules, while off-diagonal quadrants show the predicted alignment error between different molecules. Expected position error is measured in Å and is indicated as depicted in green intensities the scale.

A

	β10-BtpA	β10-BtpA(TIR)	β10-BtpB	β10-BtpB(TIR)
β11-MyD88				
β11-TIRAP				
β11-TRAM				
β11-TRIF				
MyD88-β11				
TIRAP-β11				
TRAM-β11				
TRIF-β11				

B

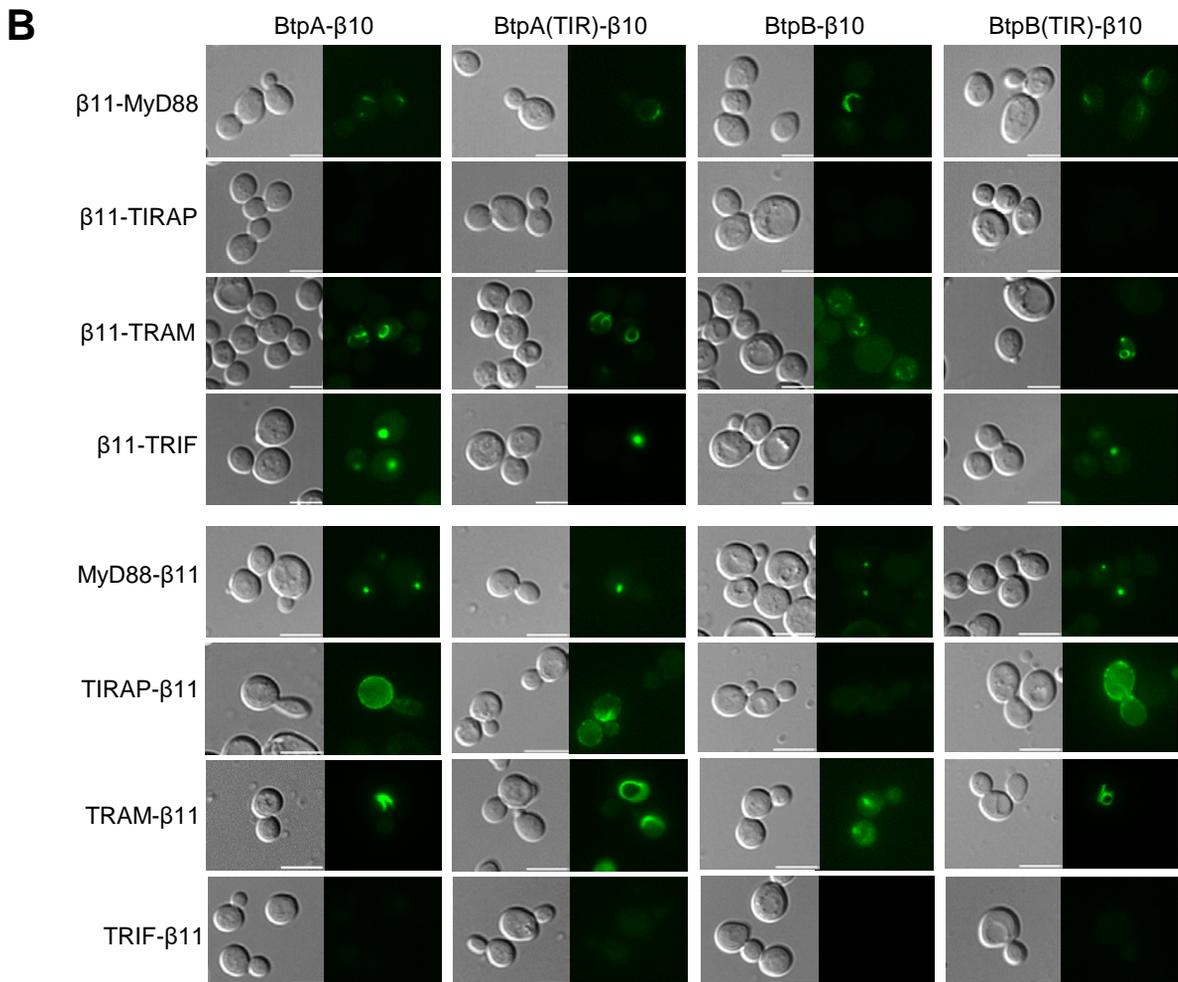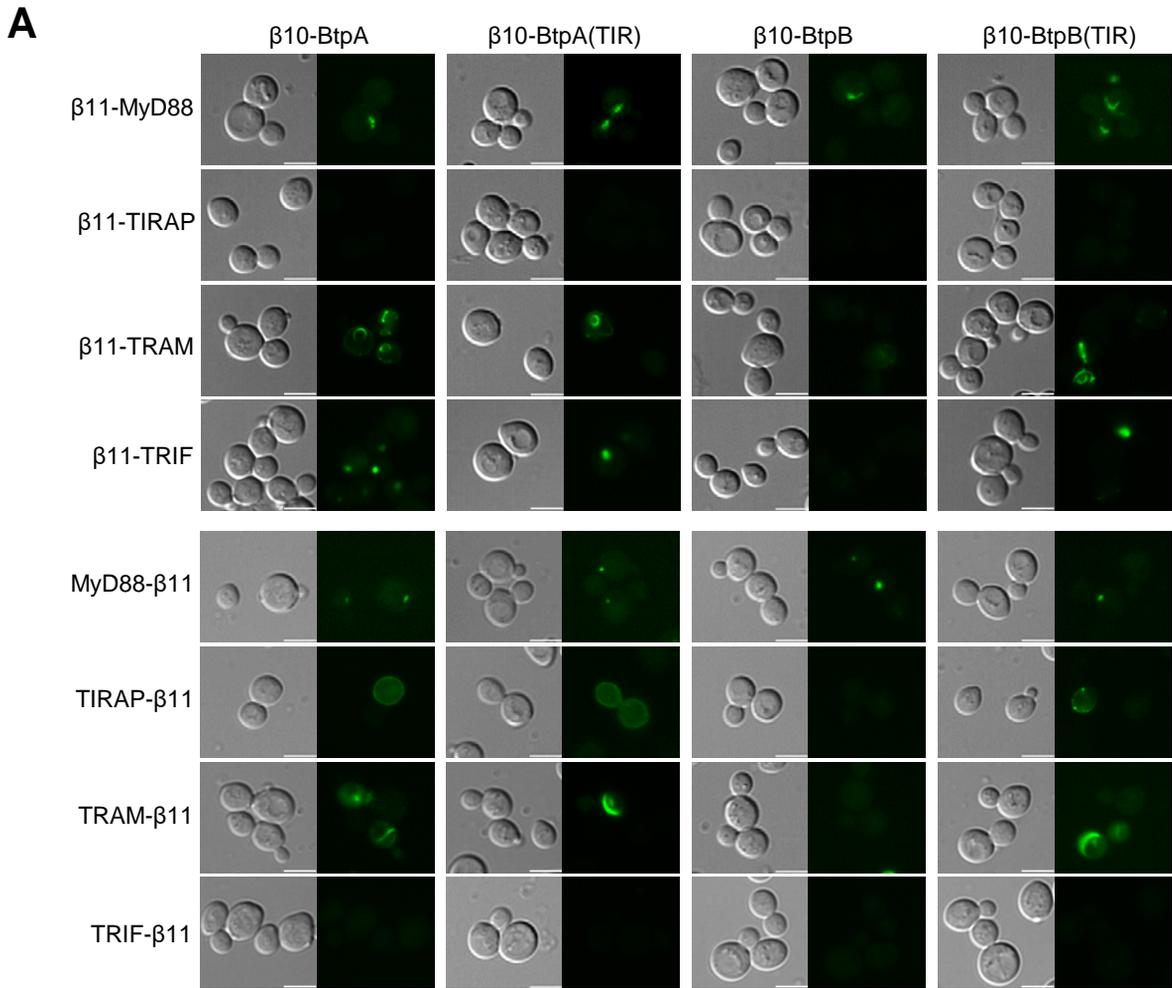|	BtpA-β10	BtpA(TIR)-β10	BtpB-β10	BtpB(TIR)-β10
β11-MyD88				
β11-TIRAP				
β11-TRAM				
β11-TRIF				
MyD88-β11				
TIRAP-β11				
TRAM-β11				
TRIF-β11				

Figure S4

Supplementary Figure S4. Representative microscopy images of all combinations of full-length Btp and Btp(TIR) with human TIR adaptors. Representative bright field and their corresponding green fluorescence images of all combinations of BtpA, BtpA(TIR), BtpB or BtpB(TIR) either N-terminally (**A**) or C-terminally (**B**) tagged with GFP β10, with the indicated fusions of GFP β11 to MyD88, TIRAP, TRAM or TRIF either N-terminally (upper half panels) or C-terminally (lower half panels). In all cases the point mutations BtpA^{E217A} or BtpB^{E234A} were present to elude TIR toxicity in yeast. The AF1 *S. cerevisiae* strain was co-transformed with the combination of GFP β10 and β11 strand plasmids, as indicated, incubated for 4 h in SG medium, and observed at the fluorescence microscope. Bars represent 5 μm.
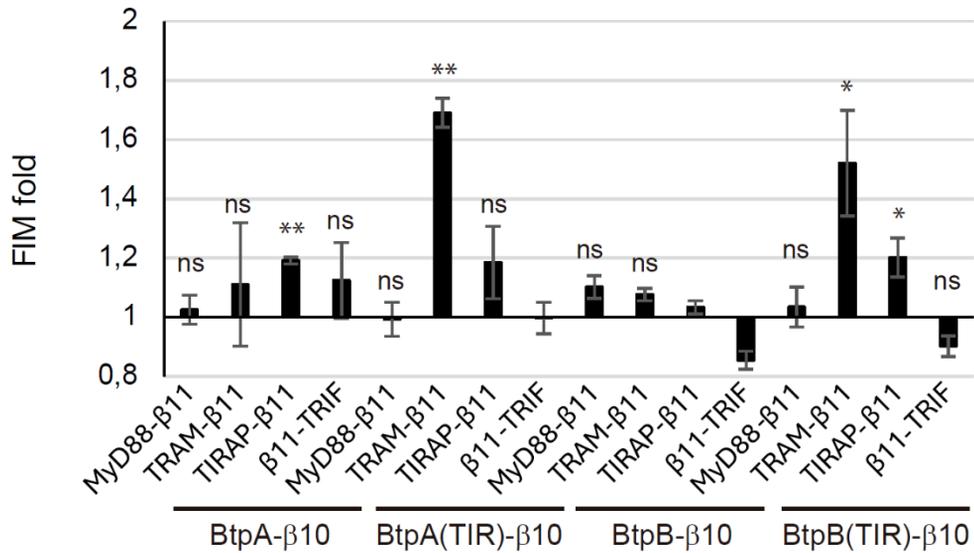
Supplementary Figure S5. Flow cytometry data of combinations of full-length Btp and Btp(TIR) with human TIR adaptors. Fluorescence intensity mean normalized to that of the negative control bearing the empty vectors (FIM fold) as derived from flow cytometry histograms of the same combinations as in Supplementary Figure S4B. Data are the average of three experimental replicates on three different transformant clones. Error bars represent the standard deviation (SD). Asterisks indicate a p value <0.05 (*) or <0.01 (**) according to the Student's t test, while 'ns' stands for non-significant.
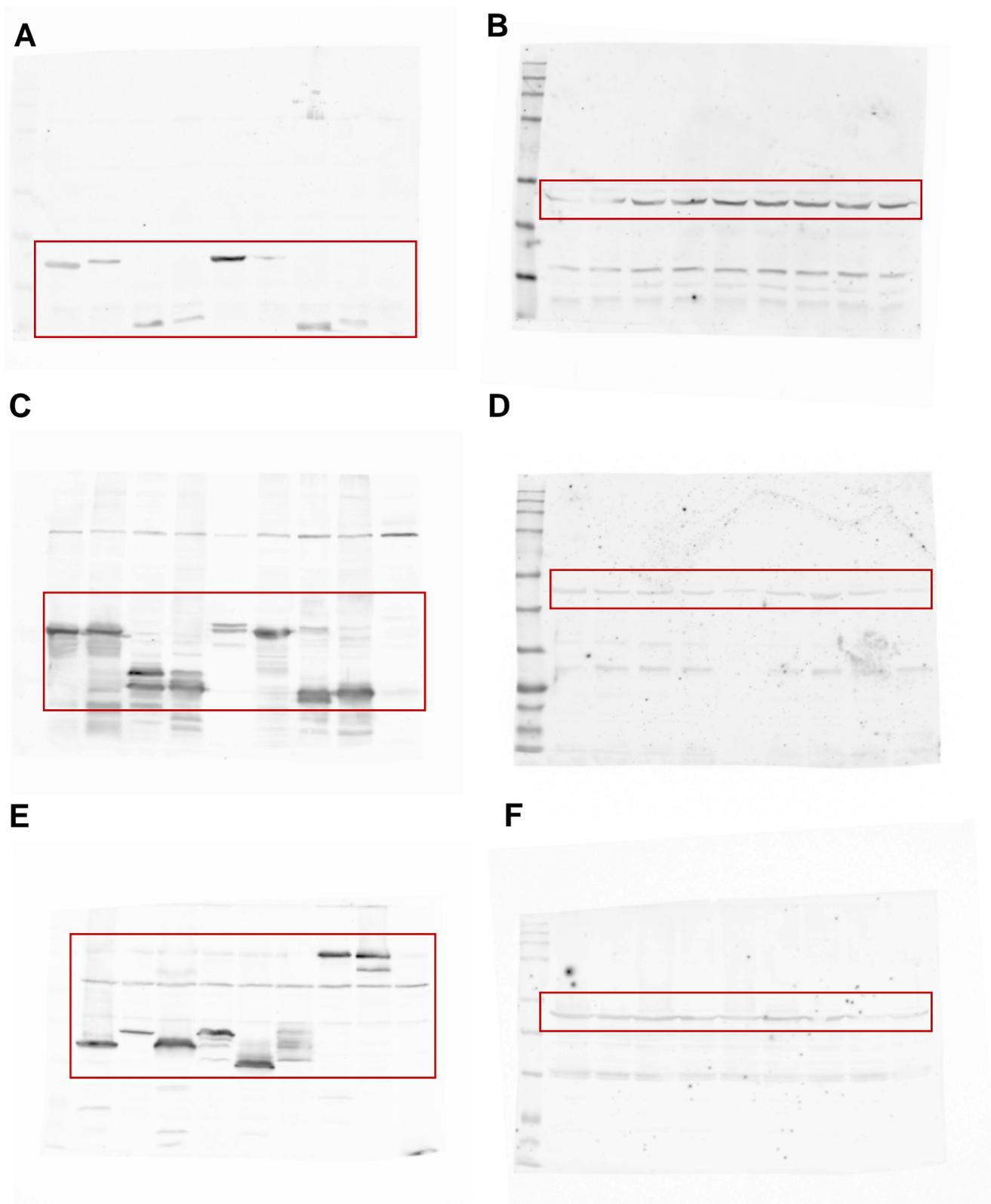
Supplementary Figure S6. Original immunoblots used for Supplementary Figure 1. (A) Source image of Figure S1A, top panel; primary antibody: anti-6×His. Secondary antibody: anti-mouse IRDye 800CW. **(B)** Source image of Figure S1A, bottom panel; primary antibody: anti-G6PDH; secondary antibody: anti-rabbit IRDye 680CW. **(C)** Source image of Figure S1B, top panel; primary antibody: anti-FLAG; secondary antibody: anti-mouse IRDye 800CW. **(D)** Source image of Figure S1B, bottom panel; primary antibody: anti-G6PDH; secondary antibody: anti-rabbit IRDye 680CW. **(E)** Source image of Figure S1C, top panel; primary antibody: anti-6xHis; secondary antibody: anti-Mouse IRDye 800CW. **(F)** Source image of Figure S1C, bottom panel; primary antibody: anti-G6PDH; secondary antibody: anti-Rabbit IRDye 680CW. Cropped areas are squared in red.

Supplementary Table S1. Plasmids used in this work and their characteristics.

Plasmid	Description	Reference
pGF-IVL794(GFP1-9)	pRS315 plasmid containing GFP1-9; Kan^R	Finnigan *et al.* (2016)
pP3a-GFPβ10	MoClo part plasmid 3a containing GFP β-strand 10	This work
pP4a-GFPβ10	MoClo part plasmid 4a containing GFP β-strand 10	This work
pP3a-GFPβ11	MoClo part plasmid 3a containing GFP β-strand 11	This work
pP4a-GFPβ11	MoClo part plasmid 4a containing GFP β-strand 11	This work
pP3b-BtpA^{E217A}	MoClo part plasmid 3b containing BtpA^{E217A}	This work
pP3b-BtpA(TIR)^{E217A}	MoClo part plasmid 3b containing BtpA(TIR)^{E217A}	This work
pP3b-BtpB^{E234A}	MoClo part plasmid 3b containing BtpB^{E234A}	This work
pP3b-BtpB(TIR)^{E234A}	MoClo part plasmid 3b containing BtpB(TIR)^{E234A}	This work
pP3b-MyD88	MoClo part plasmid 3b containing MyD88	This work
pP3b-TRAM	MoClo part plasmid 3b containing TRAM	This work
pP3b-TIRAP	MoClo part plasmid 3b containing TIRAP	This work
pP3b-TRIF	MoClo part plasmid 3b containing TRIF	This work
pP3b-3xFLAG-6xHis	MoClo part plasmid 3b containing 3xFLAG-6xHis	This work
pU316-β10-3xFLAG-6xHis	MoClo expression plasmid containing 3xFLAG-6xHis with a N-terminal β10 tag	This work
pU313-β11-3xFLAG-6xHis	MoClo expression plasmid containing 3xFLAG-6xHis with a N-terminal β11 tag	This work
pU316-β10-BtpA^{E217A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpA^{E217A} with a N-terminus β10 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU316-3xFLAG-6xHis-BtpA^{E217A}-β10	MoClo expression plasmid containing BtpA^{E271A} with a N-terminus tag 3xFLAG-6xHis and a C-terminus β10 tag	This work
pU316-β10-BtpA(TIR)^{E217A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpB(TIR)^{E234A} with	This work

	a N-terminus β10 tag and a C-terminus 3xFLAG-6xHis tag	
pU316-3xFLAG-6xHis-BtpA(TIR)^{E217A}-β10	MoClo expression plasmid containing BtpA^{217A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β10 tag	This work
pU316-β10-BtpB^{E234A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpB^{E234A} with a N-terminus β10 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU316-3xFLAG-6xHis-BtpB^{E234A}-β10	MoClo expression plasmid containing BtpB^{E234A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β10 tag	This work
pU316-β10-BtpB(TIR)^{E234A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpB(TIR)^{E234A} with a N-terminus β10 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU316-3xFLAG-6xHis-BtpB(TIR)^{E234A}-β10	MoClo expression plasmid containing BtpB(TIR)^{E234A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β10 tag	This work
pU313-β11-BtpA^{E217A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpA^{E217A} with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-BtpA^{E217A}-β11	MoClo expression plasmid containing BtpA^{E217A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-BtpA(TIR)^{E217A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpA(TIR)^{E217A} with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-BtpA(TIR)^{E217A}-β11	MoClo expression plasmid containing BtpA(TIR)^{E217A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-BtpB^{E234A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpB^{E234A} with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-BtpB^{E234A}-β11	MoClo expression plasmid containing BtpB^{E234A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-BtpB(TIR)^{E234A}-3xFLAG-6xHis	MoClo expression plasmid containing BtpB(TIR)^{E234A} with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work

pU313-3xFLAG-6xHis-BtpB(TIR)^{E234A}-β11	MoClo expression plasmid containing BtpB(TIR)^{E234A} with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-MyD88-3xFLAG-6xHis	MoClo expression plasmid containing MyD88 with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-MyD88-β11	MoClo expression plasmid containing MyD88 with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-TRAM-3xFLAG-6xHis	MoClo expression plasmid containing TRAM with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-TRAM-β11	MoClo expression plasmid containing TRAM with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-β11-TIRAP-3xFLAG-6xHis	MoClo expression plasmid containing TIRAP with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-TIRAP-β11	MoClo expression plasmid containing TIRAP with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work
pU313-β11-TRIF-3xFLAG-6xHis	MoClo expression plasmid containing TRIF with a N-terminus β11 tag and a C-terminus 3xFLAG-6xHis tag	This work
pU313-3xFLAG-6xHis-TRIF-β11	MoClo expression plasmid containing TRIF with a N-terminus 3xFLAG-6xHis tag and a C-terminus β11 tag	This work

Supplementary Table S2. Oligonucleotides used in this work and their usage.

Oligonucleotide	Sequence	Utility
GFPHOFw	GTGAAAAAGCACATCGATTATTTGATACCCCTTTGGGTTAATTACTGTTGAGGTCTTTCGGATTAGAAGCCGCCGAG	Integration of GFP barrel 1-9
GFPHORv	CCTACGCTCAGGGCACTGTACTGCCCGTGCCTGCGATGAGATACATCAATTTAAACAGTATAGCGACCAGCATTC	Integration of GFP barrel 1-9
FLAGHISMoFw	GCATCGTCTCATCGGTCTCATTCTATGGATTACAAGGACGATGATG	Used to construct pU316-β10-3xFLAG6xHIS and pU313-β11-3xFLAG6xHIS

FLAGHISMoRv	ATGCCGTCTCAGGTCTCAGGATCCCCGTGATGATGATGATG	Used to construct pU316-β10-3xFLAG6xHIS and pU313-β11-3xFLAG6xHIS
BtpA3bFw	GCATCGTCTCATCGGTCTCATTCTATGAGTTCGTACTCTTCTAATATTG	Used to construct pU316-β10-BtpA-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpA-β10, pU313-β11-BtpA-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpA-β11
BtpA3bRv	ATGCCGTCTCAGGTCTCAGGATCCGATAAGGGAATGCAGTTCTTTC	Used to construct pU316-β10-BtpA-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpA-β10, pU313-β11-BtpA-3xFLAG6xHIS, pU313-3xFLAG6xHIS-BtpA-β11, pU316-β10-BtpA(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpA(TIR)-β10, pU313-β11-BtpA(TIR)-3xFLAGG6xHIS and pU313-3xFLAG6xHIS-BtpA(TIR)-β11
BtpATIR3bFw	GCATCGTCTCATCGGTCTCATTCTGAAGCTTTCATCAAACAATCATTG	Used to construct pU316-β10-BtpA(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpA(TIR)-β10, pU313-β11-BtpA(TIR)-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpA(TIR)-β11
BtpB3bFw	GCATCGTCTCATCGGTCTCATTCTATGTACAATTTATTTGTTTCGG	Used to construct pU316-β10-BtpB-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB-β10, pU313-β11-BtpB-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpB-β11
BtpB3bRv	ATGCCGTCTCAGGTCTCAGGATCCGGTGATGAGGGCGACG	Used to construct pU316-β10-BtpB-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB-β10, pU313-β11-BtpB-3xFLAG6xHIS, pU313-3xFLAG6xHIS-BtpB-β11, pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAG6xHIS, pU313-3xFLAG6xHIS-BtpB(TIR)-β11, pU316-β10-BtpB(TIR)S162P-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)162P-β10, pU316-β10-BtpB(TIR)Y225C-

		3xFLAG6xHIS and pU316-3xFLAG6xHIS-BtpB(TIR)Y225C-β10
BtpBTIR3bFw	GCATCGTCTCATCGGTCTCATTCTCCGTCGTGGACGCGAC	Used to construct pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAGG6xHIS, pU313-3xFLAG6xHIS-BtpB(TIR)-β11, pU316-β10-BtpB(TIR)S162P-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)162P-β10, pU316-β10-BtpB(TIR)Y225C-3xFLAG6xHIS and pU316-3xFLAG6xHIS-BtpB(TIR)Y225C-β10
BtpBBsaFw	GCATCGTCTCATCTATCTTTTCCCGGTGAGGC	Used to construct pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpB(TIR)-β11
BtpBBsaRv	ATGCCGTCTCATAGACCGACGTCAAAATCGTGC	Used to construct pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpB(TIR)-β11
BtpBBsmFw	GCATCGTCTCAGCTAGACGGTTCAATCCTTCTG	Used to construct pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpB(TIR)-β11
BtpBBsmRv	ATGCCGTCTCATAGCATCCACGTAGCCGTCCG	Used to construct pU316-β10-BtpB(TIR)-3xFLAG6xHIS, pU316-3xFLAG6xHIS-BtpB(TIR)-β10, pU313-β11-BtpB(TIR)-3xFLAG6xHIS and pU313-3xFLAG6xHIS-BtpB(TIR)-β11
MyD883bFw	GCATCGTCTCATCGGTCTCATTCTATGGCTGCAGGAGGTCC	Used to construct pP3b-MyD88, pU313-β11-MyD88-3xFLAG6xHIS, pU313-3xFLAG6xHIS-MyD88-β11

MyD883bRv	ATGCCGTCTCAGGTCTCAGGATCCGGGCAGGGACAAGGCC	Used to construct pP3b-MyD88, pU313-β11-MyD88-3xFLAG6xHIS, pU313-3xFLAG6xHIS-MyD88-β11
TIRAP3bFw	GCATCGTCTCATCGGTCTCATTCTATGGCATCATCGACCTCC	Used to construct pU313-β11-TIRAP-3xFLAG6xHIS and pU313-3xFLAG6xHIS-TIRAP-β11
TIRAP3bRv	ATGCCGTCTCAGGTCTCAGGATCCAAGTAGATCAGATACTGTAGC	Used to construct pU313-β11-TIRAP-3xFLAG6xHIS and pU313-3xFLAG6xHIS-TIRAP-β11
TRAM3bFw	GCATCGTCTCATCGGTCTCATTCTATGGGTATCGGGAAGTC	Used to construct pU313-β11-TRAM-3xFLAG6xHIS, pU313-3xFLAG6xHIS-TRAM-β11
TRAM3bRv	ATGCCGTCTCAGGTCTCAGGATCCGGCAATAAATTGTCTTTGTAC	Used to construct pU313-β11-TRAM-3xFLAG6xHIS, pU313-3xFLAG6xHIS-TRAM-β11
TRIF3bFw	GCATCGTCTCATCGGTCTCATTCTATGGCCTGCACAGGCC	Used to construct pU313-β11-TRIF-3xFLAG6xHIS, pU313-3xFLAG6xHIS-TRIF-β11
TRIF3bRv	ATGCCGTCTCAGGTCTCAGGATCCTTCTGCCTCCTGCGTC	Used to construct pU313-β11-TRIF-3xFLAG6xHIS, pU313-3xFLAG6xHIS-TRIF-β11