Supplement Figures

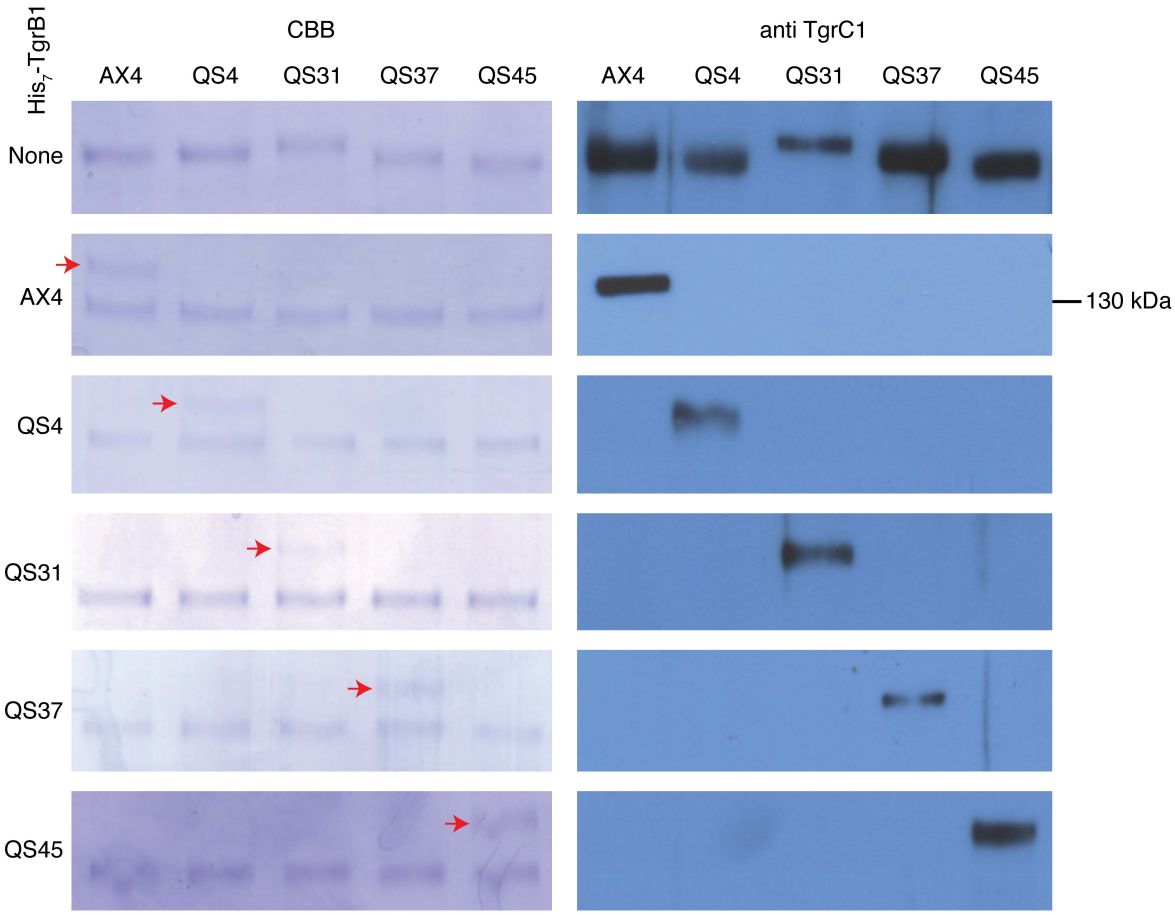

Figure S1

Figure S1. Binding of TgrB1 and TgrC1 is allele specific

We expressed the extracellular domains of TgrB1 conjugated to a His_7 tag and of untagged TgrC1 from 5 different strains, AX4, QS4, QS31, QS37 and QS45, in *Dictyostelium* cells such that the proteins were secreted into the medium. We purified the proteins, incubated each of the five His_7-TgrB1 proteins with each of the five TgrC1 proteins and pulled down the His_7-TgrB1 protein. We resolved the proteins by SDS-PAGE and visualized them by Coomassie Brilliant Blue staining (CBB) and by Western blot analysis with anti-TgrC1 antibodies as indicated above each column. The top panels in each column show the purified TgrC1 proteins without the addition of TgrB1 (None). The next 5 panels show the results of the pull-down experiments and the allotype of the His_7-TgrB1 protein is indicated on the left. The red arrows point to TgrC1 on the CBB stained gels.

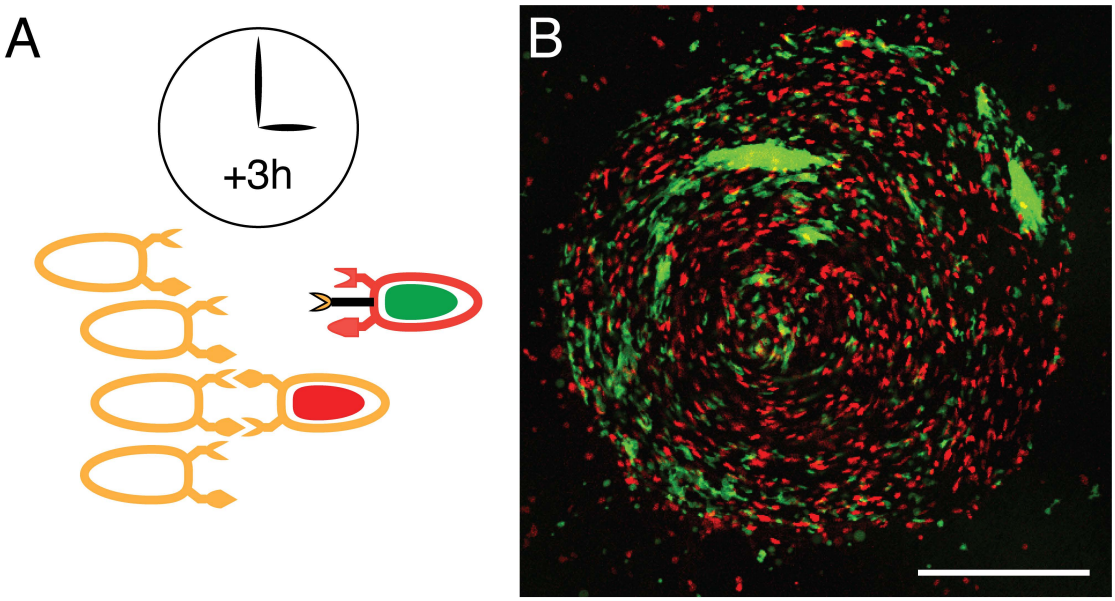

Figure S2

Figure S2. Gradual segregation despite expression of compatible TgrB1

Segregation of cells under conditions shown in Fig. 1D. A. See legend for Fig. 1A. Cells were allowed to develop for 3 additional hours. B. See legend for Fig. 1D. Bar – 0.3mm.

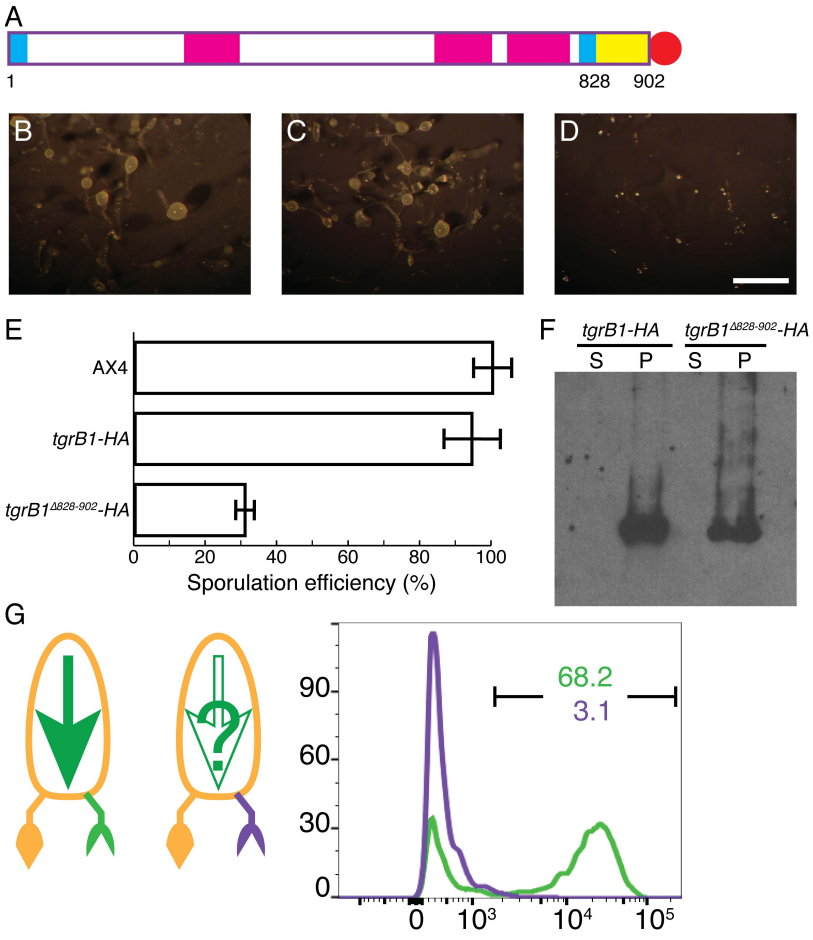

Figure S3

Figure S3. Deletion of the TgrB1 cytoplasmic domain

A. Schematic representation of HA-tagged TgrB1. Cyan bars represent the signal peptide at the N-terminus and the transmembrane domain near the C-terminus; magenta bars represent the IPT/TIG domains and the yellow bar represents the cytoplasmic domain (aa 828-902), which was deleted as indicated. Amino-acid numbers are shown below the illustration, which is drawn to scale. The red circle at the C-terminus represents an added HA-epitope (not to scale). We compared the terminal developmental morphologies of AX4 cells (B) with AX4 cells in which we replaced the resident *tgrB1* allele with *tgrB1-HA* (C) or *tgrB1*$^{\Delta 828\text{-}902}$*-HA* (D). Bar – 1mm. E. We measured the respective sporulation efficiencies (x-axis, percentage of cells that became spores; data are means ± s.e.m. of 3 independent replications) of the same

strains. F. Cells expressing intact HA-tagged TgrB1 or the respective Δ828-902 allele, as indicated, were disrupted and fractionated by ultracentrifugation. Proteins in the supernatants (S) and pellets (P) were separated by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with anti-HA-epitope antibodies. G. Cooperative differentiation: we developed pure populations of the gene-replacement strains. Ovoids represent cells; the protrusions represent TgrB1 and TgrC1, and the colors represent intact (green) and Δ828-902 (purple) proteins. Full green arrow inside the cells – *cotB*-GFP expression; empty arrow – no expression. We evaluated GFP fluorescence levels by flow cytometry and plotted the results as histograms. The x-axis represents fluorescence intensify (arbitrary units) and the y-axis the number of events. The black bar indicates the GFP-positive populations and the numbers indicate the respective fractions (%) of GFP-positive cells.

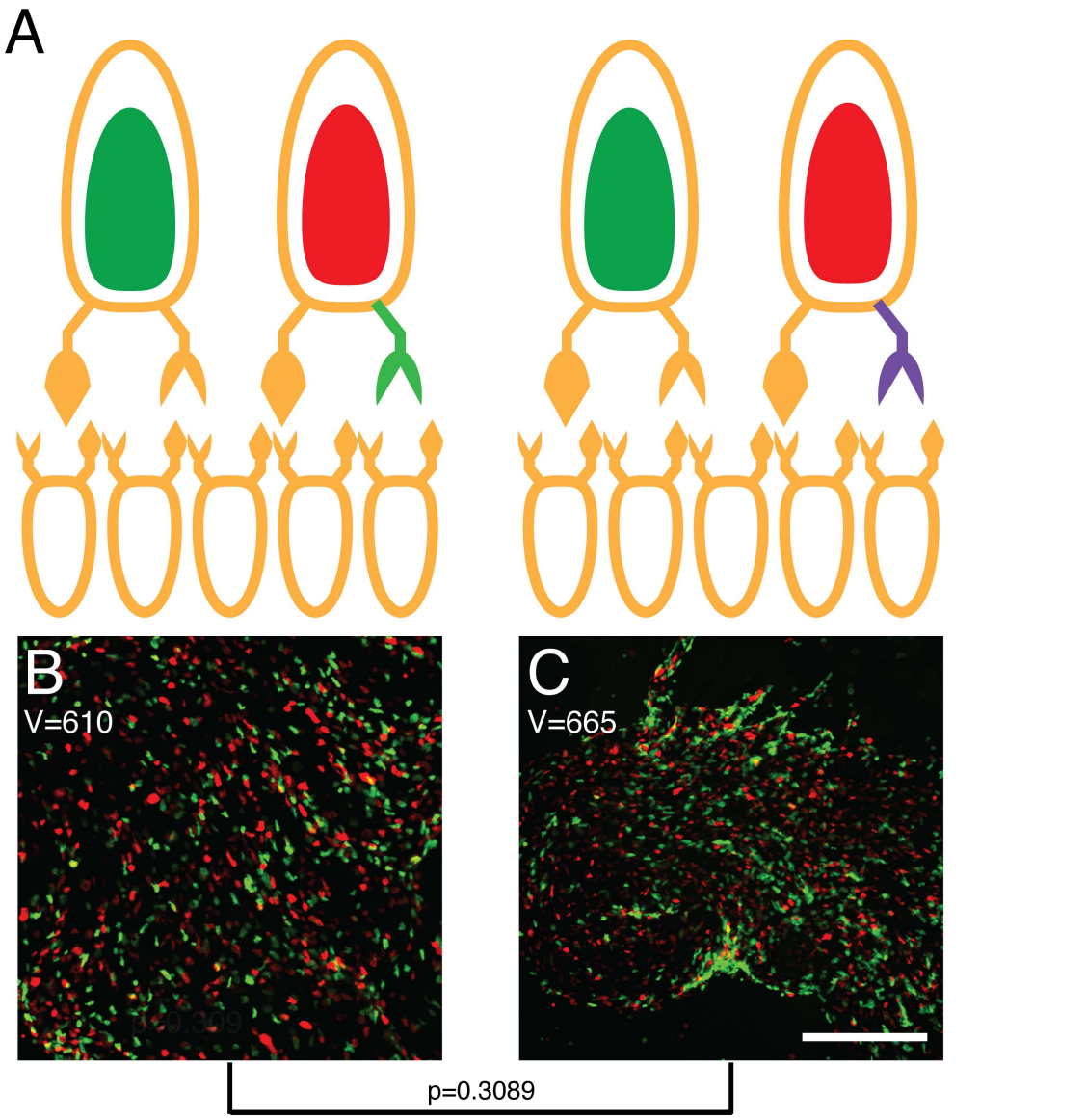

Figure S4

Figure S4. The cytoplasmic domain of TgrB1 is required for development

A. Ovoids represent cells, protrusions represent TgrB1 and TgrC1, colors represent TgrB1 alleles: wild type (AX4, tan), intact-HA tagged (green), and ∆828-902-HA tagged (purple). The larger, single cells on top represent the minority and the smaller, bottom cells represent the majority strains. Cooperative aggregation: we developed mixes of 90% unlabeled AX4 cells with 5% RFP-labeled AX4 and 5% GFP-labeled cells in which we replaced the resident *tgrB1* allele with *tgrB1-HA* (B) or *tgrB1*$^{\Delta 828\text{-}902}$*-HA* (C). The spatial distribution variance (V) is shown inside each frame. Comparison between the variances is shown below the images with a *p*-value calculated from *F*-tests. Bar – 0.3mm. D. Cooperative differentiation: we developed mixes of 0.2% TgrB1-replacement cells carrying *cotB*-GFP with 99.8% unlabeled AX4 cells. Ovoids represent cells; the protrusions represent TgrB1 and TgrC1, and the colors represent intact (green) and ∆828-902 (purple) proteins. Full green arrow inside the cells – *cotB*-GFP expression; empty arrow – no expression. We evaluated GFP fluorescence levels by flow cytometry and plotted the results as histograms. The x-axis represents fluorescence intensify (arbitrary units) and the y-axis the number of events. The black bar indicates the GFP-positive populations and the numbers indicate the respective fractions (%) of GFP-positive cells.

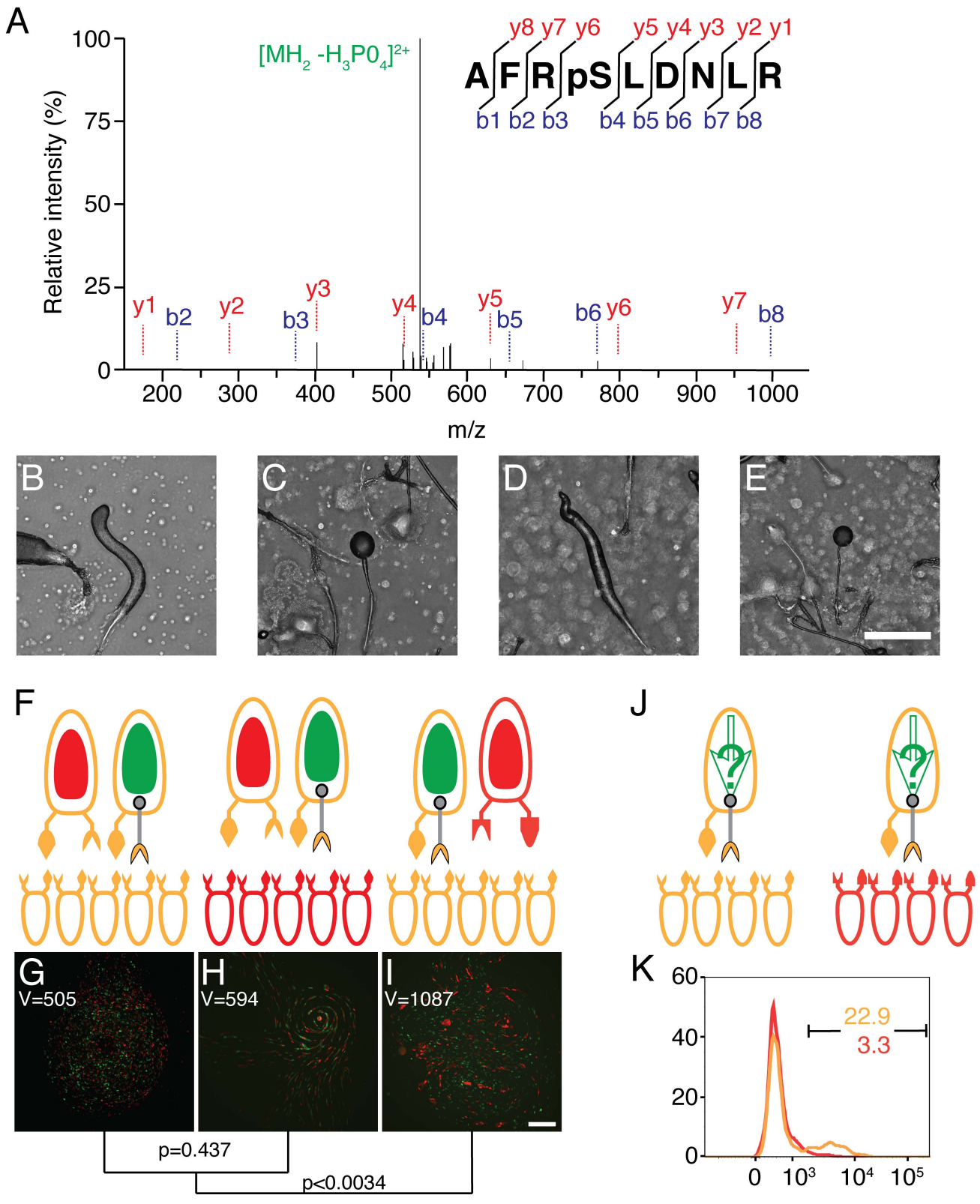

Figure S5

Figure S5. Physical and phenotypic analysis of TgrB1 phosphorylation

A. We analyzed the phosphopeptides associated with TgrB1^{myc2AX4} by liquid chromatography tandem-mass spectrometry of samples collected at 12-14 hours of development. The mass-to-charge ratio (m/z) is plotted on the x-axis and the relative intensity (%) on the y-axis. Black bars represent the mass spectra that scored higher than 2%, red and blue dashed lines indicate the predicted fragmentation pattern of the only tryptic peptide that showed a significant positive score (amino acids 842-850, as indicated on the upper right hand of the chart), and green text indicates the corresponding loss-of-H_3PO_4 peptide. The developmental morphologies of wild-type cells (B – 16h, C – 36h) and mutant *tgrB1*$^{-}$*tgrB1*^{myc2AX4S845A} cells (D – 16h, E – 36h) are shown after development on buffered agar. Bar – 0.5mm. F. Ovoids represent cells, protrusions represent TgrB1 and TgrC1 proteins, and colors represent allotypes: tan – AX4, red – *tgrB1C1*^{QS31}. Grey protrusions represent the mutant *tgrB1*^{AX4S845A} allele. Internal green and red ovoids represent constitutive GFP and RFP expression, respectively. The larger, single cells on top represent the minority and the smaller, bottom cells represent the majority strains. Cooperative aggregation: we developed mixes of 90% unlabeled AX4 cells (G, I) or *tgrB1C1*^{QS31} (H) with 5% RFP-labeled AX4 (G, H) or *tgrB1C1*^{QS31} (I) cells and 5% GFP-labeled *tgrB1*^{AX4S845A} cells. The spatial distribution variance (V) is shown inside each frame. Comparisons between the variances are shown below the images with *p*-values calculated from *F*-tests. Bar – 0.2mm. J. Cooperative differentiation: we developed mixes of 0.2% *tgrB1*^{AX4S845A} cells *cotB*-GFP cells with 99.8% unlabeled cells of different allotypes: tan – AX4, red – *tgrB1C1*^{QS31}. The empty green arrow inside the ovoid represents the *cotB*-GFP expression under investigation. We evaluated GFP fluorescence levels by flow cytometry and plotted the results as a histogram (K). The x-axis represents fluorescence intensify (arbitrary units) and the y-axis the number of events. The black bar inside the histogram indicate the GFP-positive population and the respectively colored numbers indicate the fraction (%) of GFP-positive cells.

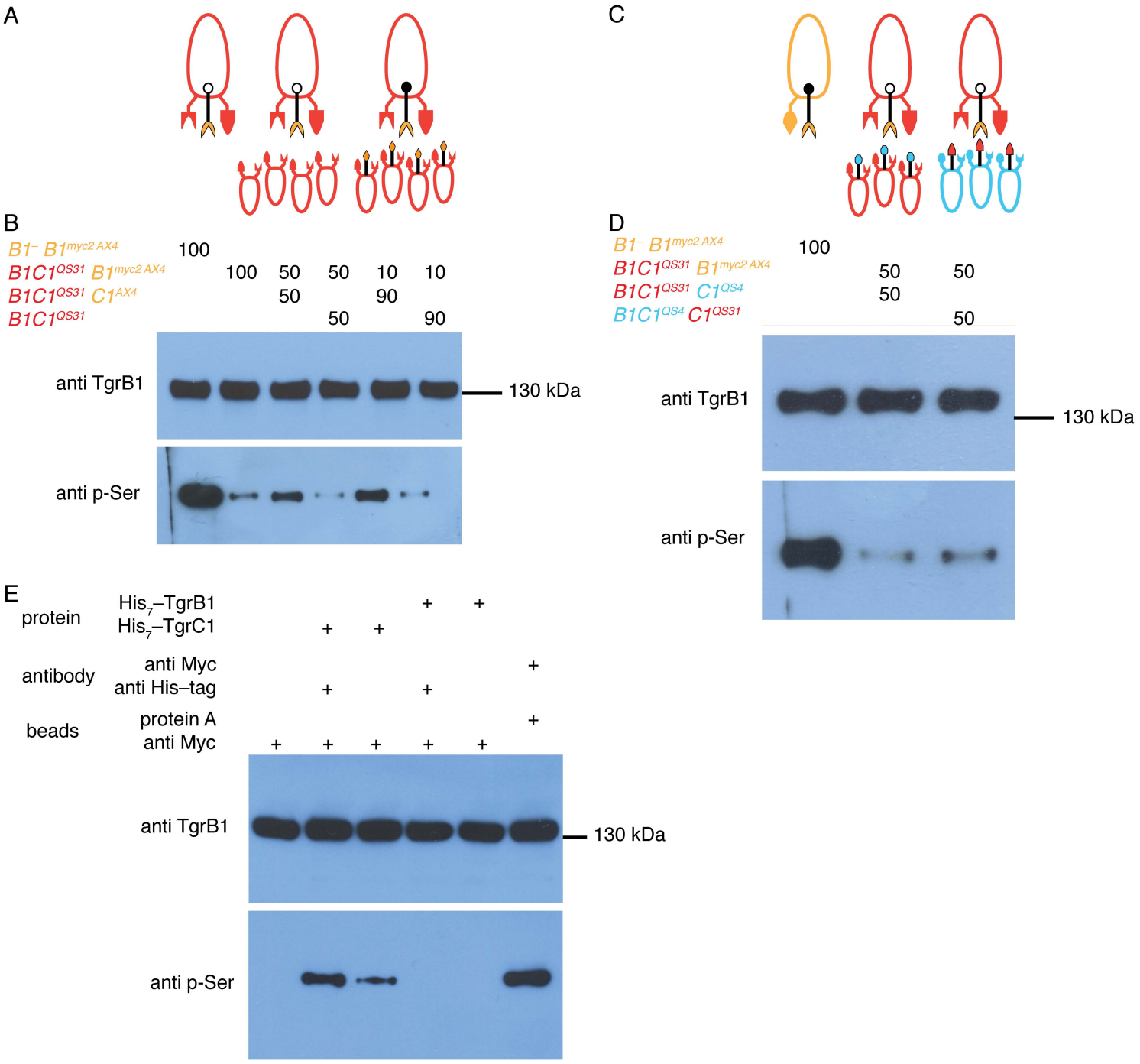

Figure S6

Figure S6. TgrC1-induction of TgrB1 phosphorylation: allele specificity and induction by soluble ligand

A, B – We repeated the experiments described in Figure 5C and 5D, but used the *tgrB1C1*^{QS31} background (red) instead of the *tgrB1C1*^{QS4} background (blue) to evaluate specificity with a second set of alleles. C – we generated strains that carried two *tgrC1* alleles, both of which are incompatible with the *tgrB1*^{AX4} allele: *tgrB1C1*^{QS31}*tgrC1*^{QS4} (red ovals and protrusions – *tgrB1C1*^{QS31}, black and blue protrusion – *tgrB1*^{QS4}) and *tgrB1C1*^{QS4}*tgrC1*^{QS31} (blue ovals and protrusions – *tgrB1C1*^{QS4}, black and red protrusion – *tgrB1*^{QS31}). D – We mixed each of these strains at equal proportions, as indicated, with *tgrB1C1*^{QS31}*tgrB1*^{myc2AX4} cells (red ovals and protrusions – *tgrB1C1*^{QS31}, black and tan protrusion – *tgrB1*^{myc2AX4}), developed them for 12 hours and tested for phosphorylation of the myc-tagged TgrB1 protein as in Fig. 5. We used a pure population of the complementation strain *tgrB1*$^{-}$*tgrB1*^{myc2AX4} (tan ovals and protrusion – AX4, black and tan protrusion – *tgrB1*^{myc2AX4}) as a positive control. E. We introduced the *tgrB1*^{myc2} allele into *tgrB1*-null *tgrC1*-null cells, developed the cells for 14 hours, dissociated them and incubated them in suspension with purified His_7-TgrB1 or His_7-TgrC1 protein, as indicated above the image, or with buffer as a control. We also included mouse-anti-Myc or mouse-anti His-tag antibodies as indicated. After incubation, we lysed the cells and pulled down the proteins with protein A-beads or with beads conjugated to anti-Myc antibodies as indicated. We resolved the proteins by gel electrophoresis and performed Western blot analyses with antibodies against TgrB1 or against phosphorylated Serine as indicated on the left. The position of the nearest molecular weight marker is indicated on the right.

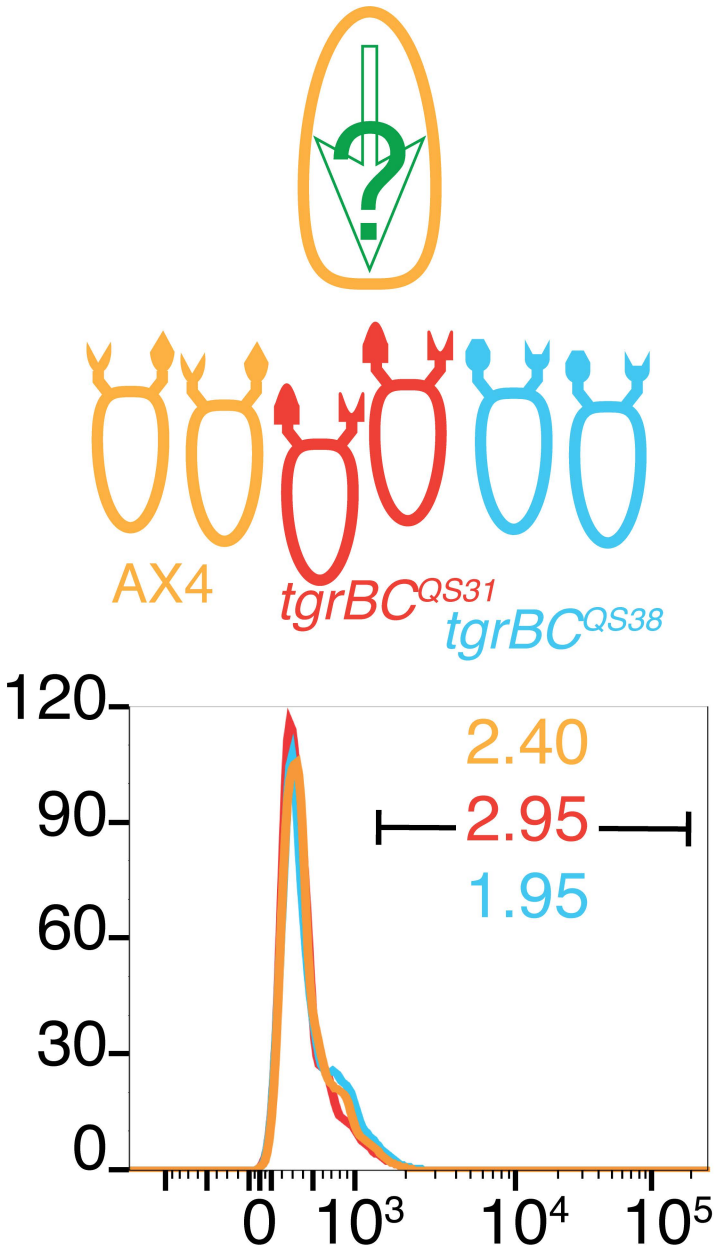

Figure S7

Figure S7. The *tgrB1-C1* deletion strain does not express *cotB*-GFP

We developed mixes of 0.2% *tgrB1*$^{-}$*tgrC1*$^{-}$ cells carrying *cotB*-GFP and 99.8% unlabeled cells of different allotypes: tan – AX4, red – *tgrB1C1*^{QS31}, blue – *tgrB1C1*^{QS38}. In the illustration, ovoids represent cells, protrusions represent TgrB1 and TgrC1 proteins. The larger, single cell on top represent the minority and the smaller, bottom cells represent the majority strains (each tested separately). The empty green arrow inside the cell represents lack of *cotB*-GFP expression. We evaluated GFP fluorescence levels by flow cytometry and plotted the results as histograms. The x-axis represents fluorescence intensify (arbitrary units) and the y-axis the number of events. The black bar indicates the GFP-positive populations and the numbers indicate the respective fractions (%) of GFP-positive cells. The majority cells are as indicated by the line colors.

Supplement Table S1. Dictyostelium discoideum strains used

Relevant genotype	Reference
AX4-RFP	Hirose *et al.*, 2011
AX4-GFP	Hirose *et al.*, 2011
tgrB1^{-}	Benabentos *et al.*, 2009
*tgrB1*Δ-GFP	Benabentos *et al.*, 2009
tgrC1^{-}	Benabentos *et al.*, 2009
tgrB1^{-}*tgrC1*$^{-}$	Hirose *et al.*, 2011
tgrB1C1^{QS4}	Hirose *et al.*, 2011
tgrB1C1^{QS31}	Hirose *et al.*, 2011
tgrB1C1^{QS38}	Hirose *et al.*, 2011
tgrB1C1^{QS31}*GFP*	Hirose *et al.*, 2011
tgrB1C1^{QS31}*tgrB1*^{AX4}*-GFP*	This work
tgrB1C1^{QS31}*tgrC1*^{AX4}*-GFP*	This work
tgrB1C1^{QS31}*tgrB1*^{AX4}	This work
tgrB1C1^{QS31}*tgrC1*^{AX4}	This work
AX4-RFP[cotB/sfGFP]	Hirose *et al.*, 2015
AX4tgrB1^{QS31}*-RFP[cotB/sfGFP]*	This work
AX4tgrC1^{QS31}*-RFP[cotB/sfGFP]*	This work
tgrB1-HA	This work
tgrB1$^{\Delta 828\text{-}902}$*-HA*	This work
tgrB1-HA[cotB/GFP]-RFP	This work
tgrB1$^{\Delta 828\text{-}902}$*-HA[cotB/GFP]-RFP*	This work
tgrB1-HA-GFP	This work
tgrB1$^{AX4\Delta 828\text{-}902}$*-HA-GFP*	This work
tgrB1C1^{QS31}*tgrB1*^{AX4}*-HA-RFP*	This work

tgrB1C1^{QS31}tgrB1$^{AX4\Delta828\text{-}902}$-HA-RFP	This work
tgrB1C1^{QS31}tgrB1^{AX4}-HA[cotB/sfGFP]-RFP	This work
tgrB1C1^{QS31}tgrB1$^{AX4\Delta828\text{-}902}$-HA[cotB/sfGFP]-RFP	This work
tgrB1^{-}tgrC1^{-}tgrB1$^{AX4\text{-}G275D}$	This work
tgrB1^{-}tgrC1^{-}tgrB1$^{AX4\text{-}G307D}$	This work
tgrB1^{-}tgrC1^{-}tgrB1$^{AX4\text{-}L846F}$	This work
AX4tgrB1$^{AX4\text{-}G275D}$[cotB/sfGFP]-RFP	This work
AX4tgrB1 $^{AX4\text{-}G307D}$[cotB/sfGFP]-RFP	This work
AX4tgrB1$^{AX4\text{-}L846F}$[cotB/sfGFP]-RFP	This work
tgrB1^{-}myc$_2$tgrB1^{AX4}	This work
tgrB1^{-}tgrC1^{-}myc$_2$tgrB1^{AX4}	This work
tgrB1C1^{QS4}myc$_2$tgrB1^{AX4}	This work
tgrB1C1^{QS31}myc$_2$tgrB1^{AX4}	This work
tgrB1^{-}myc$_2$tgrB1(S845A)^{AX4}	This work
AX4 act15::tgrC1^{AX4}	This work
AX4 act15::tgrC1^{QS4}	This work
AX4 act15::tgrC1^{QS31}	This work
AX4 act15::tgrC1^{QS37}	This work
AX4 act15::tgrC1^{QS45}	This work
AX4 act15::his$_7$tgrB1^{AX4}	This work
AX4 act15::his$_7$tgrB1^{QS4}	This work
AX4 act15::his$_7$tgrB1^{QS31}	This work
AX4 act15::his$_7$tgrB1 ^{QS37}	This work
AX4 act15::his$_7$tgrB1^{QS45}	This work
tgrB1C1^{QS4}tgrC1^{AX4}	This work
tgrB1C1^{QS31}tgrC1^{AX4}	This work
tgrB1C1^{QS31}tgrC1^{QS4}	This work
tgrB1C1^{QS4}tgrC1 ^{QS31}	This work

tgrB1:tgrB1$^{AX4(L846F)}$*,bsR*	This work
tgrB1-HA/tgrC1-rep	This work
tgrB1ΔC-HA/tgrC1-rep	This work
tgrB1^{QS31}*/tgrC1*^{QS31}*-rep*	Hirose *et al.*, 2011
tgrB1^{AX4S845A}-GFP	This work
tgrB1^{AX4S845A}[*cotB*-GFP]-RFP	This work

Supplement Table S2. Key vectors used

Vector name	Reference	Comments
pDM304		GenBank Accession Number EU912539
pDXA-CFP-MCS		GenBank Accession Number AJ510160
pDXA-3C		GenBank Accession Number X851118
pLPBLP		Dictybase ID: 9
pDXA-GFP2	Levi *et al.*, 2000	http://dictybase.org/db/cgi-bin/dictyBase/SC/plasmid_details.pl?id=110
pDXA-tdTomato	Hirose *et al.*, 2011	
pDXA-tdTomato,hygR	Benabentos *et al.*, 2009	
pcotB/sfGFP	Hirose *et al.*, 2015	
tgrB1:tgrB1^{AX4},bsR	This work	
tgrC1:tgrC1^{AX4},bsR	This work	
tgrB1:tgrB1^{AX4}-HA,bsR	This work	
tgrB1:tgrB1^{AX4}ΔC-HA,bsR	This work	

tgrB1:tgrB1$^{AX4(G275D)}$,bsR	Li *et al.*, 2016	
tgrB1:tgrB1$^{AX4(G307D)}$,bsR	Li *et al.*, 2016	
tgrB1:tgrB1$^{AX4(L846F)}$,bsR	This work	
tgrB1-HA/tgrC1-rep	This work	
tgrB1ΔC-HA/tgrC1-rep	This work	
tgrB1^{QS31}/tgrC1^{QS31}-rep	Hirose *et al.*, 2011	
pDMBsr *tgrB1*::myc$_2$TgrB1	This work	
pDMBsr *tgrB1*::myc$_2$TgrB1(S845A)	This work	
pDXA *act15*::*tgrC1*^{AX4}	This work	
pDXA *act15*::*tgrC1*^{QS4}	This work	
pDXA *act15*::*tgrC1*^{QS31}	This work	
pDXA *act15*::*tgrC1*^{QS37}	This work	
pDXA *act15*::*tgrC1*^{QS45}	This work	
pDXA act15::his$_7$tgrB1^{AX4}	This work	
pDXA act15::his$_7$tgrB1^{QS4}	This work	
pDXA act15::his$_7$tgrB1^{QS31}	This work	
pDXA *act15*::*his$_7$tgrB1* ^{QS37}	This work	
pDXA act15::his$_7$tgrB1^{QS45}	This work	