Supplementary Information

Catalysis-dependent condensation of citrate synthase involved in glutamate overproduction in *Corynebacterium glutamicum*

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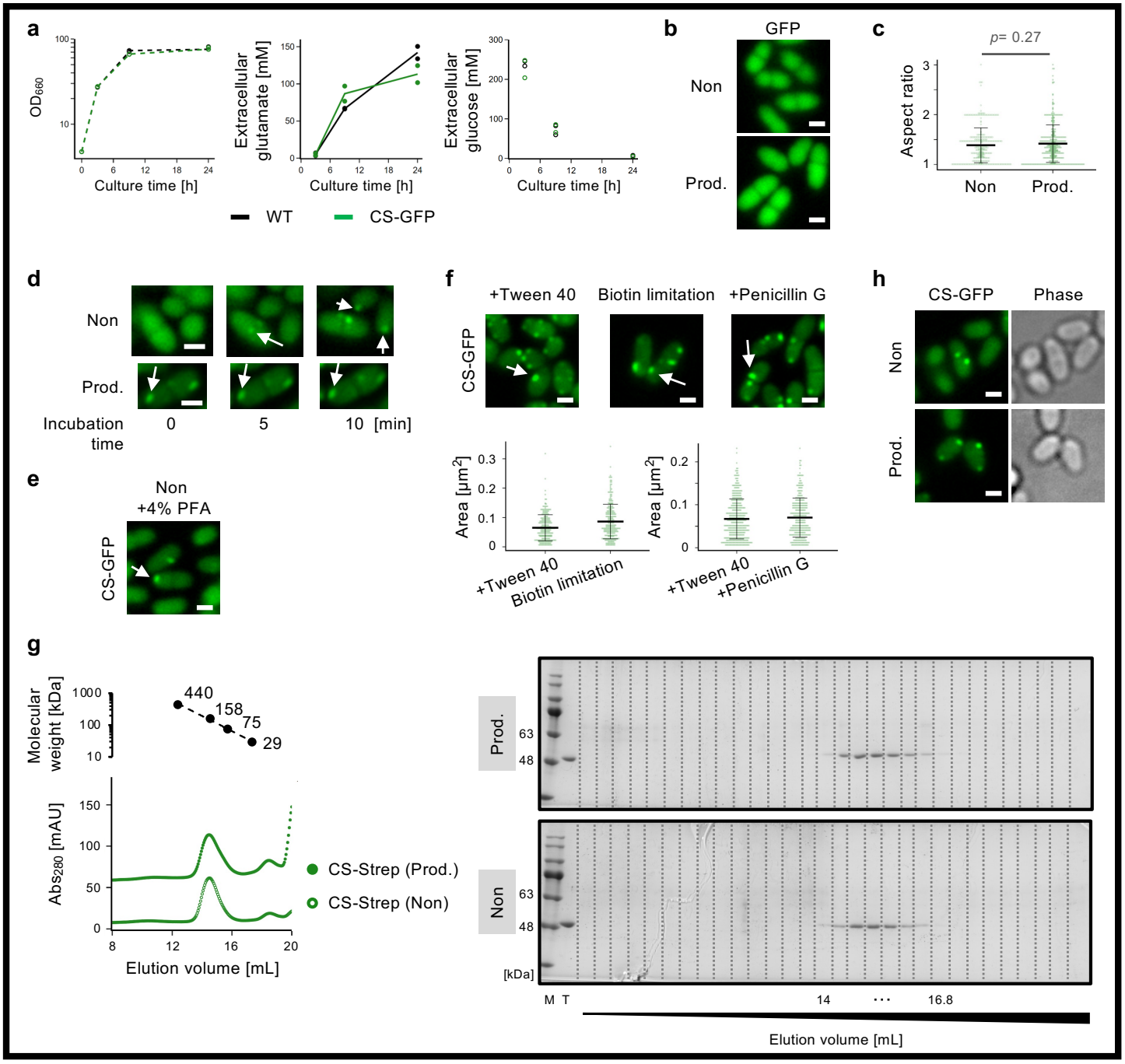
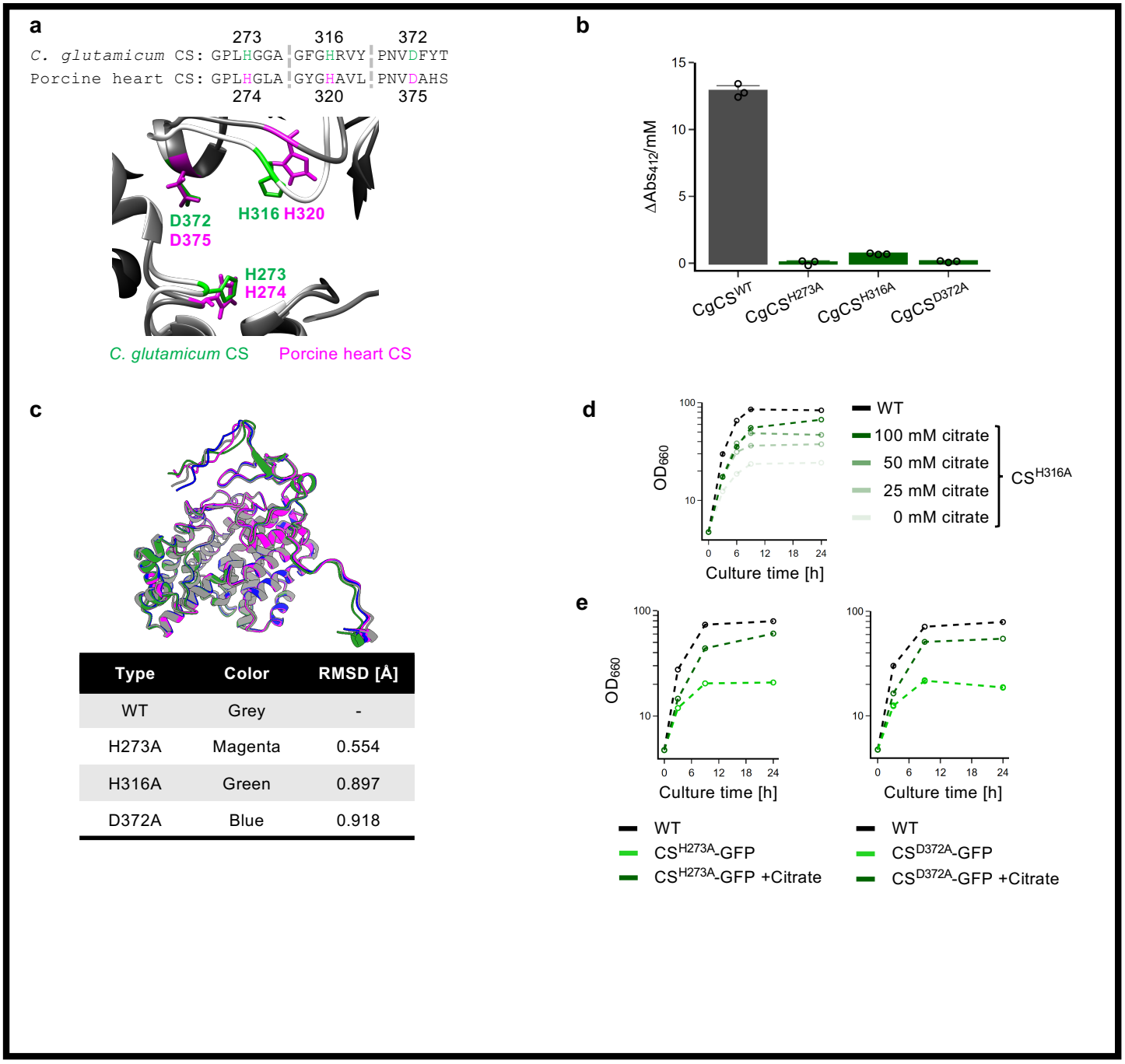


Supplementary Fig. S1 Citrate synthase formed condensates in *Corynebacterium glutamicum* cells

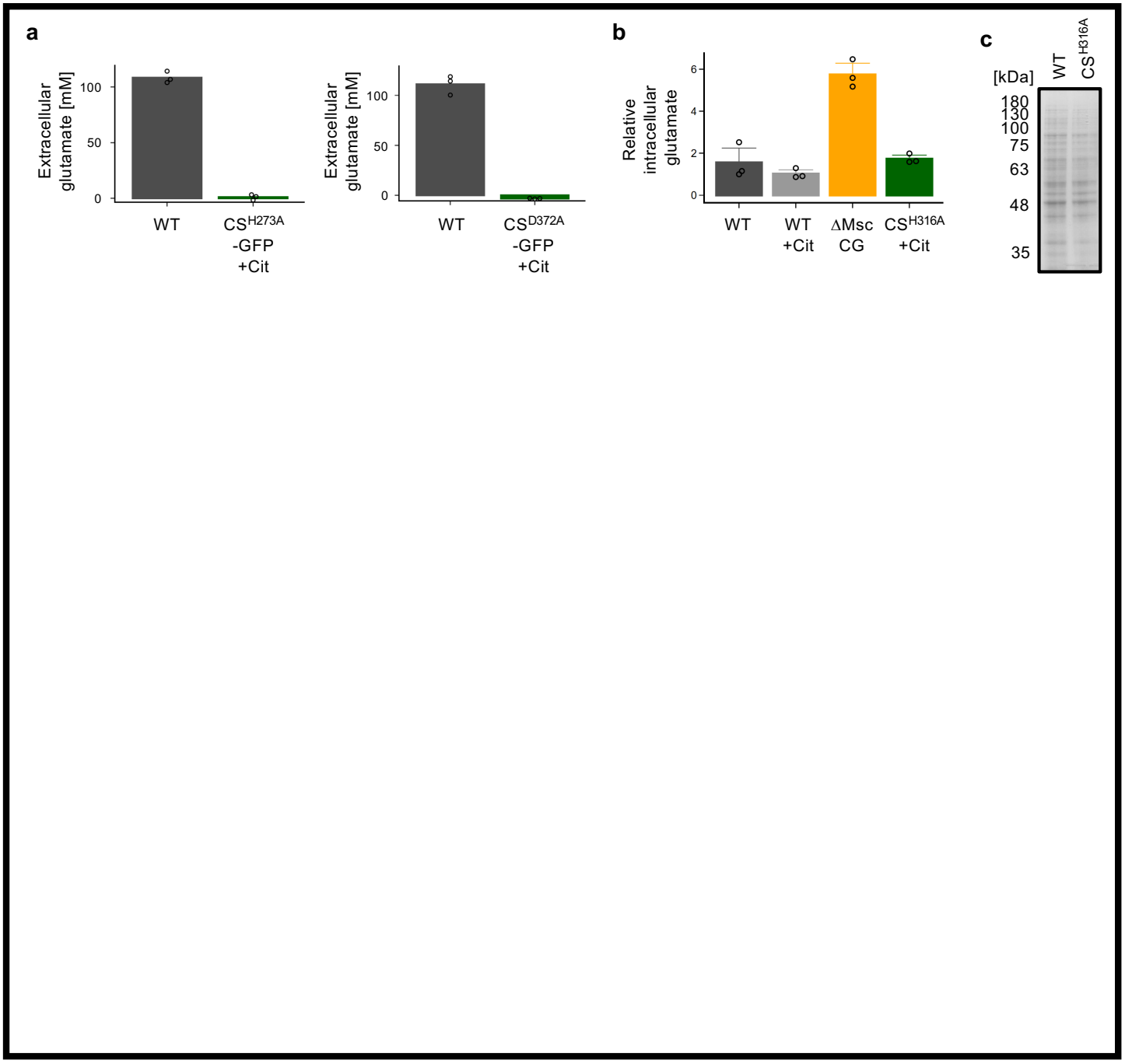
a Growth, extracellular glutamate production, and extracellular glucose consumption of the wild-type (13032) and CgCS-GFP (NMa43) strains. Two independent culture experiments were conducted. **b** Images of mEGFP expression in *C. glutamicum*. The NMa42 strain was used as a control. **c** Aspect ratios of CS condensates. The same datasets were used as shown in Figure 1d. **d** Time-lapse images of CgCS-GFP. **e** CgCS-GFP images of fixed cells using 4%(w/v) paraformaldehyde. We added 4%(w/v) paraformaldehyde to the cultures in non-producing conditions after 9 h and rotated at 31.5 ˚C for 20 min before microscopy. **f** CgCS-GFP images in *C. glutamicum* cells under glutamate-producing conditions induced by different triggers (after 9 h). **g** Size-exclusion chromatography analysis was performed using CgCS-Strep purified from *C. glutamicum* cells under glutamate-producing (Prod.) and non-producing (Non) conditions. Chromatography was performed using a Superdex 200 Increase 10/300 GL column (GE Healthcare) with buffer D at a flow rate of 0.75 mL/min, and 0.4 mL fractions were collected. The calculated molecular weights compared with the standards were 133 and 129 kDa, respectively, suggesting that they exist as dimers (50.1 kDa for monomers). **h** GFP fluorescence and bright-field images of the CS-GFP producing strain (NMa43). At least two experiments were repeated independently for **b**–**h**, and a representative dataset is shown.



Type	Color	RMSD [Å]
WT	Grey	-
H273A	Magenta	0.554
H316A	Green	0.897
D372A	Blue	0.918

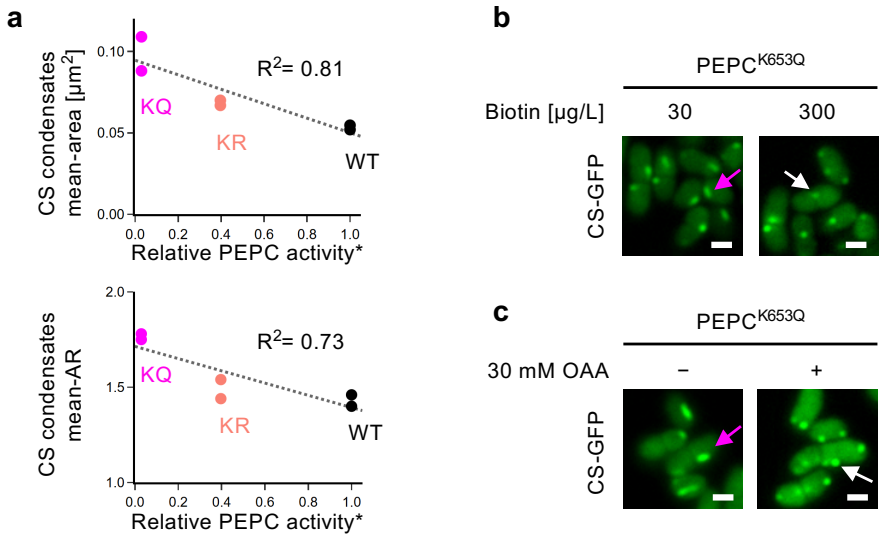
Supplementary Fig. S2 Active CS was required for condensation

a Amino acid sequence and structural alignments of *Corynebacterium glutamicum* and porcine heart CS. Structures of CgCS available in the AlphaFold Protein Structure Database (AF-P42457-F1-v4) and porcine heart CS (PDB ID: 3ENJ) were used, and structural alignment was performed using UCSF Chimera software (ver. 1.19). **b** CS activity of recombinant $CgCS^{WT}$-Strep, $CgCS^{H273A}$-Strep, $CgCS^{H316A}$-Strep, and $CgCS^{D372A}$-Strep proteins. Data from three replicate assays are presented. **c** AlphaFold modeling of $CgCS^{WT}$, $CgCS^{H273A}$, $CgCS^{H316A}$, and $CgCS^{D372A}$ structures. The root mean square deviation (RMSD) values between $CgCS^{WT}$ and variants are shown. **d** Dose-dependent restoration of the growth of CS^{H316A} strain (NMa191) by citrate. WT, ATCC 13032 strain. **e** Restoration of the growth of $CgCS^{H273A}$-GFP (NMa183) and $CgCS^{D372A}$-GFP (NMa381) strains with 100 mM citrate. Cells were cultured in a biotin-30 glutamate production medium without Tween 40 inducer; a representative data set of three biologically independent experiments is shown (**d** and **e**).



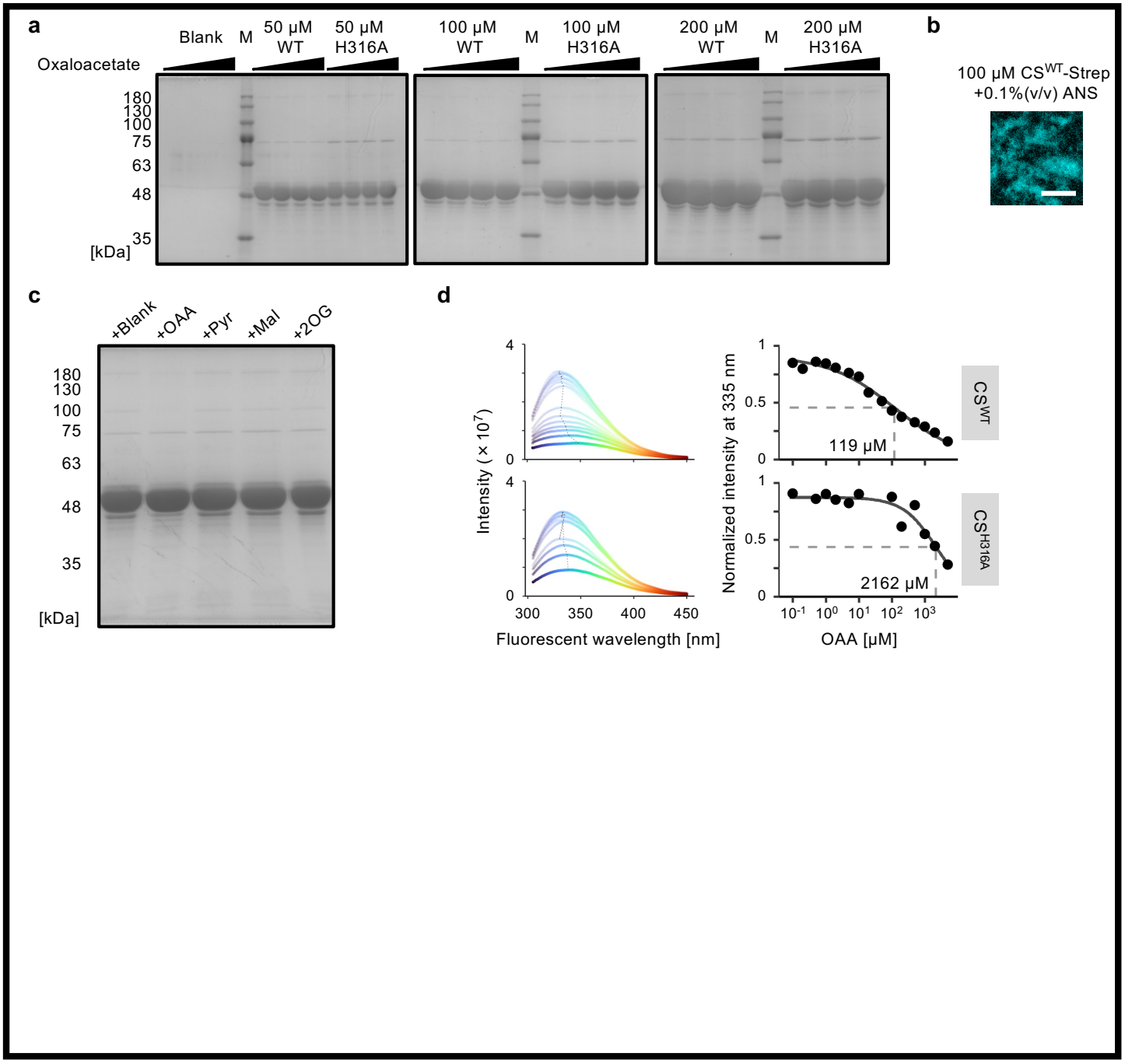
Supplementary Fig. S3 CS condensation and its relationship with glutamate production

a Extracellular glutamate production (after 24 h) of CgCS^{H273A}-GFP (NMa183) and CgCS^{D372A}-GFP (NMa381) strains under citrate (100 mM)-added glutamate-producing conditions. The WT and mutant strains were grown under normal glutamate-producing conditions. Three independent culture experiments were performed. **b** Intracellular glutamate levels in cells grown under glutamate-producing conditions (after 9 h). WT (ATCC 13032) with and without 100 mM citrate, ΔMscCG (NM80) and CgCS^{H316A} (NMa191) with 100 mM citrate. Data from three biologically independent experiments are shown. **c** CBB staining of *Corynebacterium glutamicum* cell lysates (5 μg) used for the GDH and GOGAT activity assays (loading control of Fig. **3c**).



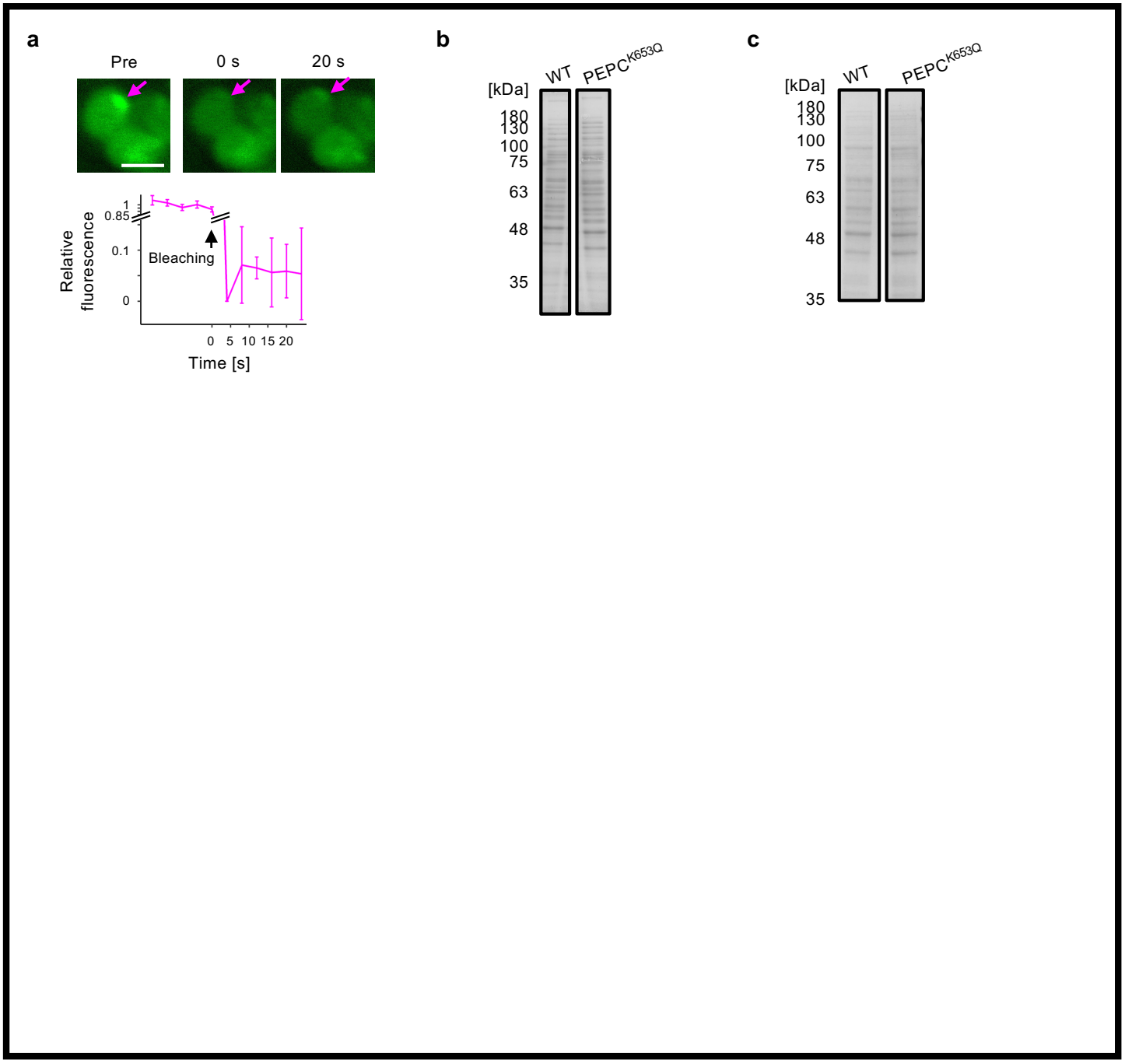
Supplementary Fig. S4 Oxaloacetate was a factor affecting CS condensation

a Correlation between size and aspect ratio of CgCS condensates and PEPC activity. PEPC activity data were obtained from our previous study[23] and are shown relative to WT activity. Circles represent the average value of >400 analyzed condensates, and data from two biologically independent experiments are plotted. Linear regression analysis was performed using R software. **b** CgCS-GFP in the PEPC^{K653Q} strain (NMa190) under normal (30 µg/L) and excess (300 µg/L) biotin-supplemented glutamate-producing conditions. **c** CgCS-GFP in PEPC^{K653Q} cells (NMa190) after dilution in buffer containing 30 mM oxaloacetate (OAA). At least two experiments were repeated independently for **b** and **c**, and a representative dataset is shown.



Supplementary Fig. S5 Substrate-assisted conformational change was important for CgCS condensation

a Loading control for recombinant CgCS^{WT}-Strep and CgCS^{H316A}-Strep used for the turbidity assays (Fig. **5a**). Proteins (50, 100, and 200 mM) were incubated with 0, 0.01, 0.1, or 1 mM oxaloacetate. M, molecular size markers. **b** 8-Anilino-1-naphthalenesulfonic acid (ANS) staining of CS^{WT}-Strep protein after 24-h of incubation in a turbidity assay. CgCS^{WT}-Strep (100 μM) was incubated with 10 μM ANS. Scale bars, 20 μm. **c** Loading control of the CgCS^{WT}-Strep protein used for turbidity assays (Fig. **5b**). **d** Intrinsic fluorescence spectra of CS^{WT}-Strep and CS^{H316A}-Strep proteins in the presence of various concentrations (0–5 mM) of oxaloacetate (OAA). A part of the data (between 305 and 360 nm fluorescence wavelength) are shown in Fig. **5c**. Dotted line indicates the peak positions of fluorescence (left). Fluorescence intensity plot at 335 nm of CS^{WT}-Strep and CS^{H316A}-Strep proteins in the presence of various concentrations (0.0001–5 mM) of OAA. The intensity was normalized to that without OAA. Fitting with a logistic function was performed using R software (right). All experiments were repeated independently at least twice, and a representative dataset is shown.



Supplementary Fig. S6 Metabolic impact of abnormal CgCS condensation

a FRAP analysis of CgCS condensates in PEPC^{K653Q} strain (NMa190). Bleaching was performed using seven independent condensates, and a representative set is shown. **b** CBB staining of cell lysates (2 µg protein) used for the CS activity assay (loading control of Fig. **6b**). **c** CBB staining of cell lysates (5 µg protein) used for GDH and GOGAT activity assays (loading control of Fig. **6c**).

Table S1 Strain list

Strain name	Description	Source
ATCC 13032	*Corynebacterium glutamicum* wild-type strain	JCM 1318
NMa43	CS-mEGFP	This study
NMa42	ATCC 13032 harboring pECt::mEGFP (pNMa60)	This study
YF1	CS-Strep	This study
NMa358	CS-GFP-FUS^{N}	This study
NMa191	CS(H316A)	This study
NMa94	CS(H316A)-GFP	This study
NMa183	CS(H273A)-GFP	This study
NMa381	CS(D372A)-GFP	This study
NMa260	NMa42 harboring pECt::CS^{WT}-mCherry (pNMa62)	This study
NMa263	NMa42 harboring pECt::CS(H316A)-mCherry (pNMa63)	This study
NM80	ΔMscCG (*cgl1270*-deleted)	This study
MY100	ΔPEPC (*cgl1585*-deleted)	[23]
MY40	PEPC(K653Q)	[23]
MY38	PEPC(K653R)	[23]
NMa240	ΔPEPC (*cgl1585*-deleted), CS-GFP	This study
NMa190	PEPC(K653Q), CS-GFP	This study
NMa211	PEPC(K653R), CS-GFP	This study

Table S2. Plasmid list

Plasmid name	Description	Use
pNMa60	pECt::mEGFP	Expression of mEGFP in *C. glutamicum*
pNMa62	pECt::CS^{WT}-mCherry	Expression of CS^{WT}-mCherry
pNMa63	pECt::CS^{H316A}-mCherry	Expression of CS^{H316A}-mCherry
pMK47	pET21a::CS^{WT}-Strep	Expression of CS^{WT}-Strep in *E. coli*
pNMa68	pET21a::CS^{H316A}-Strep	Expression of CS^{H316A}-Strep in *E. coli*

Table S3. Primer list

Primer	Sequence (5'-3')	For construction of
PNMa93	CCTCGCGAGGAGCGCGTGAGCAAGGGCGAG	NMa43
PNMa94	CTCGCCCTTGCTCACGCGCTCCTCGCGAGG	NMa43
PNMa95	CGAGCTGTACAAGTAAATTTAGCGGATGATTC	NMa43
PNMa96	GAATCATCCGCTAAATTTACTTGTACAGCTCG	NMa43
PYF382	TTCGAGCTCGGTACCCGGG	YF1, NMa43
PNMa114	CTGCAGGTCGACTCTAGAG	YF1, NMa43
PYF383	CGAACTGTGGGTGGGACCAACCAC	YF1
PYF384	TTGGTCCCACCCACAGTTCGAGAAG	YF1
PNMa101	ACCACCATGGAATTCGAGCTCGGAAAGGAATAATTACTCTAATG GTGAGCAAGGGCGAG	NMa42
PNMa102	GTCGACTCTAGAGGATCCCCGGTTACTTGTACAGCTCGTC	NMa42
PNMa313	GACGAGCTGTACAAGGCGGGTACCATGGATC	NMa358
PNMa314	GATCCATGGTACCCGCCTTGTACAGCTCGTC	NMa358
PNMa315	CAGGACCGTGGATAGATTTAGCGGATGATTC	NMa358
PNMa316	GAATCATCCGCTAAATCTATCCACGGTCCTG	NMa358
PNMa115	CTCATGGGCTTCGGAGCCCGCGTTTACAAGAAC	CS^{H316A} substitution
PNMa117	GTTCTTGTAAACGCGGGCTCCGAAGCCCATGAG	CS^{H316A} substitution
PNMa116	CTCTACCCGAACGTAGCCTTCTACACCGGCCTG	CS^{D372A} substitution
PNMa118	CAGGCCGGTGTAGAAGGCTACGTTCGGGTAGAG	CS^{D372A} substitution
PNMa221	CTGTCCGGCCCACTGGCCGGTGGCGCAAACCAG	CS^{H273A} substitution
PNMa222	CTGGTTTGCGCCACCGGCCAGTGGGCCGGACAG	CS^{H273A} substitution
PNMa172	CGGGTACCGAGCTCGCTACTTGTACAGCTC	pNMa62 and 63
PNMa126	CCATGGAATTCGAGCTCGGAGTTTTTTTCCGAA	pNMa62 and 63
PNM282	ATATGCCCTGCAGGCACCTGTCATCCGCTCAG	NM80

PNM283	CGTCTGTAATCAGCGTCGAGCCAAGATTAGCGC	NM80
PNM284	GCGCTAATCTTGGCTCGACGCTGATTACAGACG	NM80
PNM285	ATATGCCCTGCAGGCCTTGGACATCATCACGGAG	NM80
PMK91	TTTAAGAAGGAGATATACATATGTTTGAAAGGGATATCGTG	pMK47, pNMa68
PMK95	GGTGGTGGTGCTCGAGTGCTTATTACTTCTCGAACTGTGGGTG G	pMK47, pNMa68

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