Supplemental Material

“Comparative CRISPR type III - based knockdown of essential genes in hyperthermophilic *Sulfolobales* and the evasion of lethal gene silencing”

Zink *et al.*, 2020

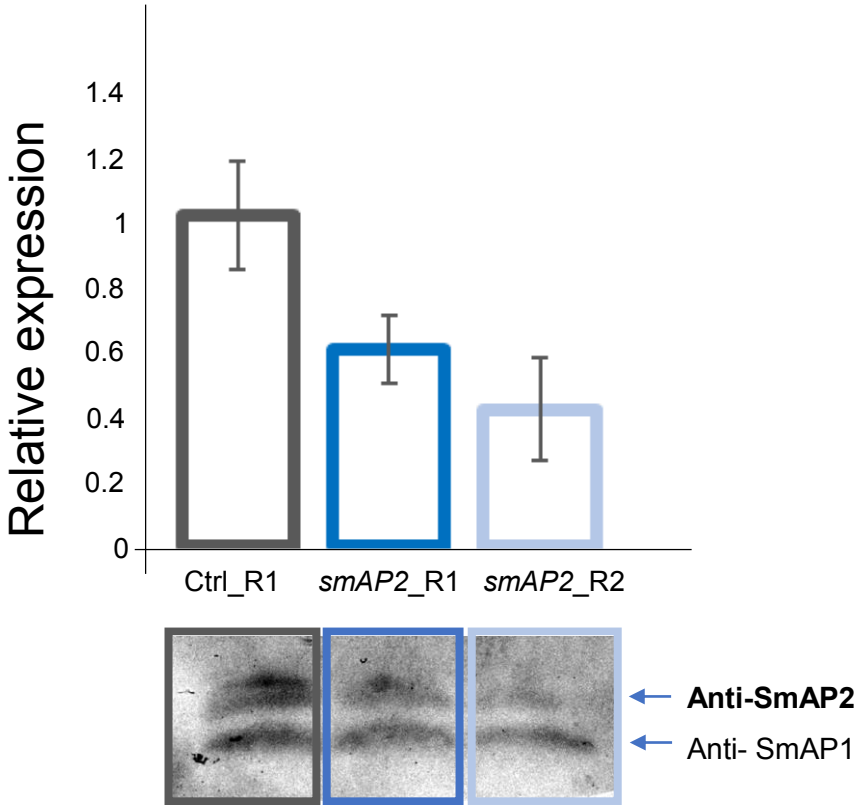
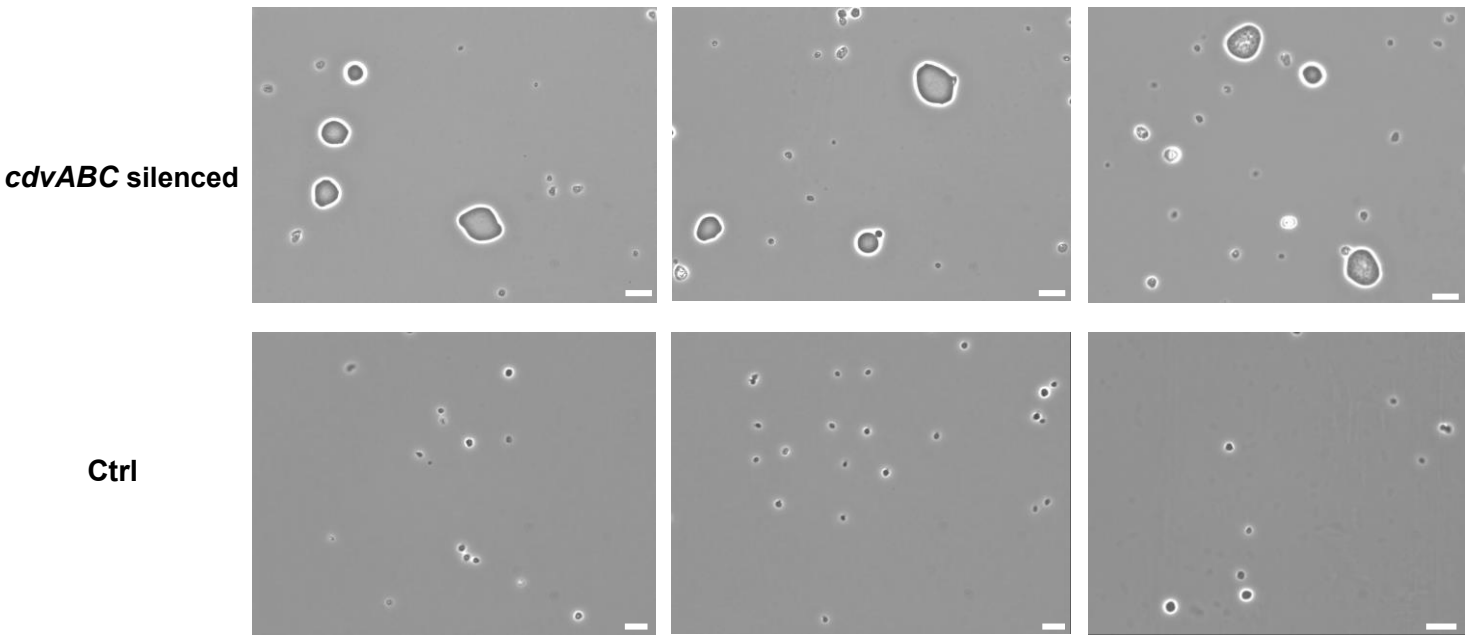


Figure S1: Silencing of *smAP2* on verified on RNA and protein level. Upper panel: Expression of the *smAP2* mRNA relative to the 16S rRNA measured by RT-qPCR on reverse transcribed RNA extracts of individual biological replicates (R1, R2). Control (dark grey left bar, Ctrl_P1); *smAP2* silencing cultures: *smAP2*_P1 (dark blue middle bar), *smAP2*_P2 (light blue right bar). Error bars, mean ± *SD* (technical replicates = 3); Lower panel: Western blot analyses of same cultures as above using Anti-SmAP2 detecting SmAP2 (upper band) and Anti-SmAP1 binding the SmAP1 proteins (loading control, lower band).

A)



B)

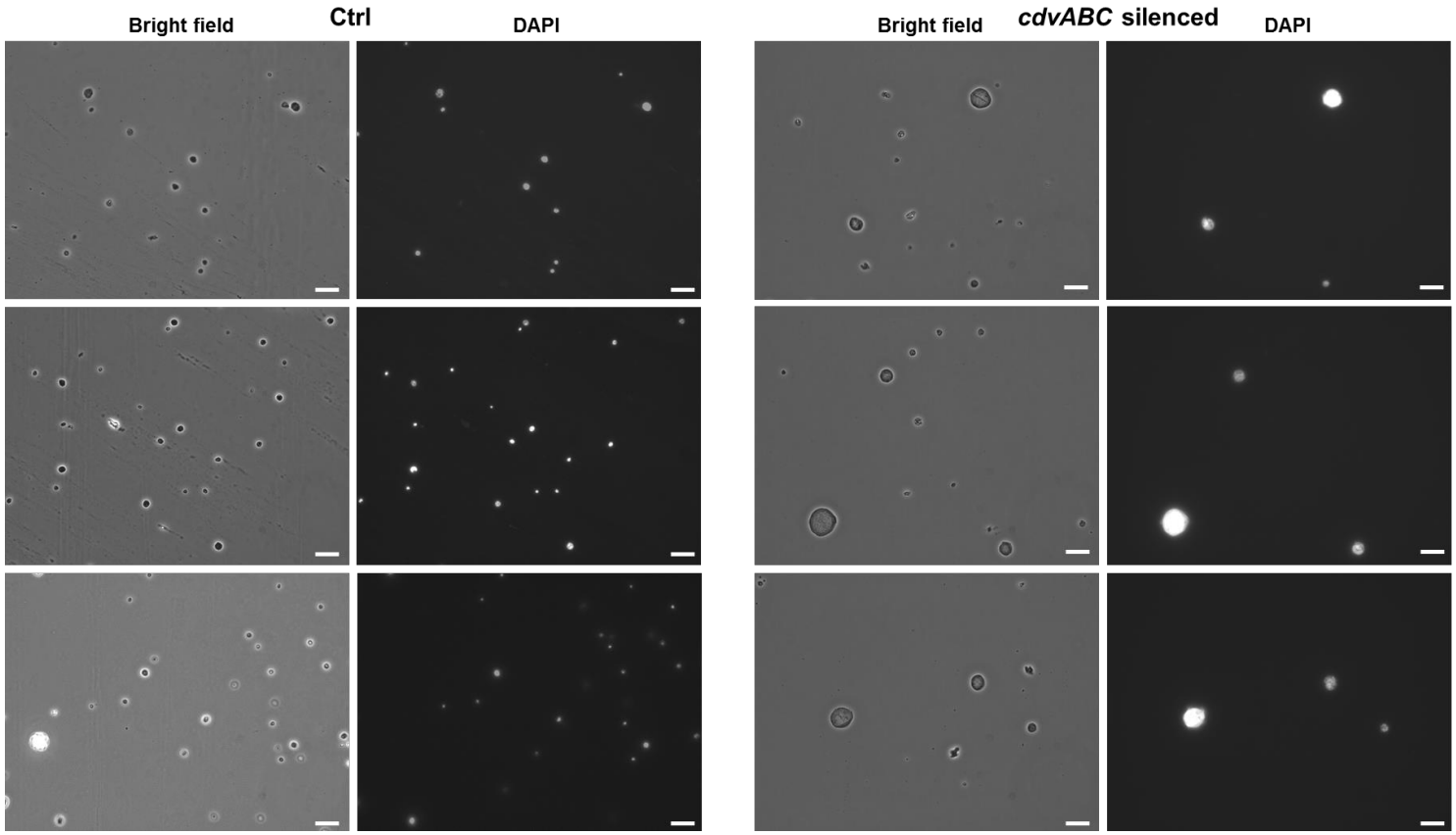


Figure S2: Heterogeneous cell sizes in *cdvA* silencing cultures. A) Phase contrast micrographs of untreated cells sampled from a growing culture (time between sampling and imaging was around 2 min). Upper panel: CdvABC-depleted culture (miniCR-CA-2 transformants), lower panel: control (miniCR-Ctrl transformants). Scale bar: 5 µm. **B)** Phase contrast and fluorescent microscopy of fixed, DAPI-stained cultures (from same cultures as in A); left panel: control cultures, right panel: CdvABC - depleted cultures. Scale bar= 5µm. Cells size are slightly decreased in comparison to fresh cultures (shown in A), potentially due to fixation and staining.

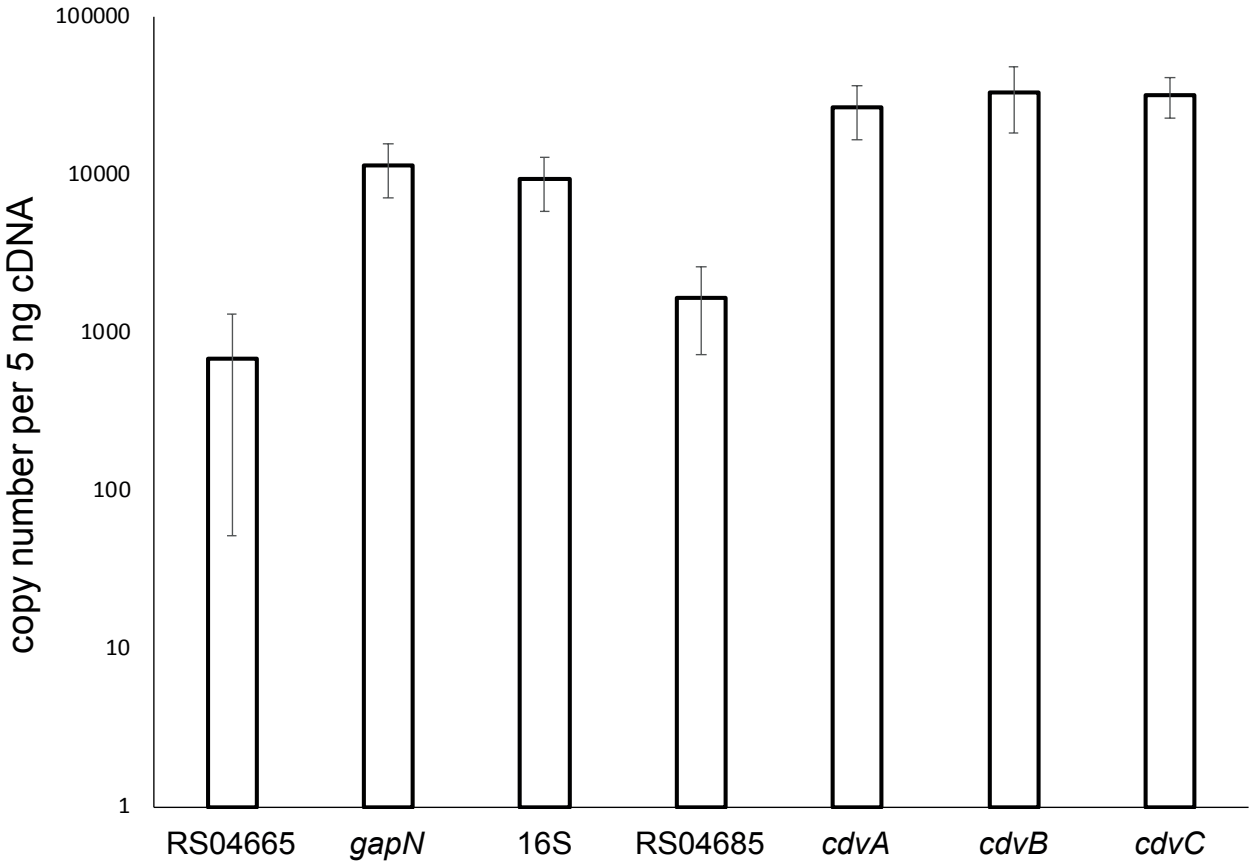


Figure S3: Transcript copy numbers of genes of the *cdv* locus, neighboring genes and reference genes. Transcript numbers of reverse - transcribed RNA from control cultures (carrying non-silencing control vector) (c.f. Fig. 2B) measured by RT-qPCR per 5ng total cDNA are shown (respective genes cloned on a plasmid were used as standard). 16S rRNA and *gapN-3* (glyceraldehyde-3-phosphate dehydrogenase) are located elsewhere in the genome, whereas RS04665 and RS04685 are located up and downstream of the *cdvABC* locus, respectively. For the upstream gene RS04665, 15 and 44 times lower number of transcripts are measured compared to *gapN*/16S and the genes of the *cdv* locus, respectively. Furthermore, higher error bars indicate stochastic transcription of RS04665. Error bars, mean ± *SD* (n = 3).

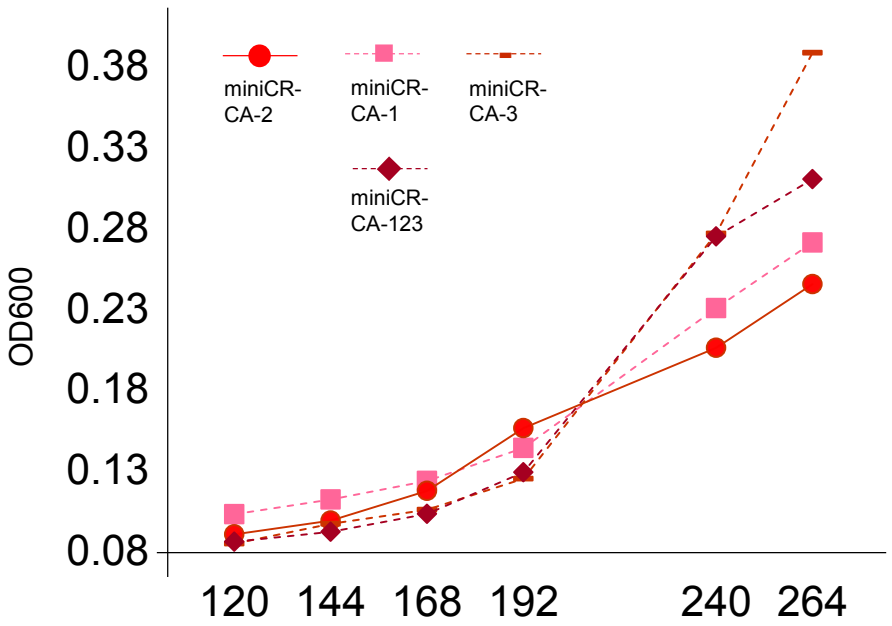


Figure S4: Stably silenced and reverting cultures in *cdvA* silencing experiments. Growth profiles (OD600) of the same miniCR-CA transfectants as in Fig. 3, with the difference that transformation solutions were directly inoculated into liquid medium after electroporation (not purified over plaque assay as their replicate cultures shown in Fig. 3, see Materials and Methods) and therefore had a longer lag-phase. Cultures transfected with strong miniCR reverted (colored discontinuous lines), leading to growth recovery at later timepoints compared to the stably-silenced miniCR-CA-2 culture (continuous lines).

A) **B)**

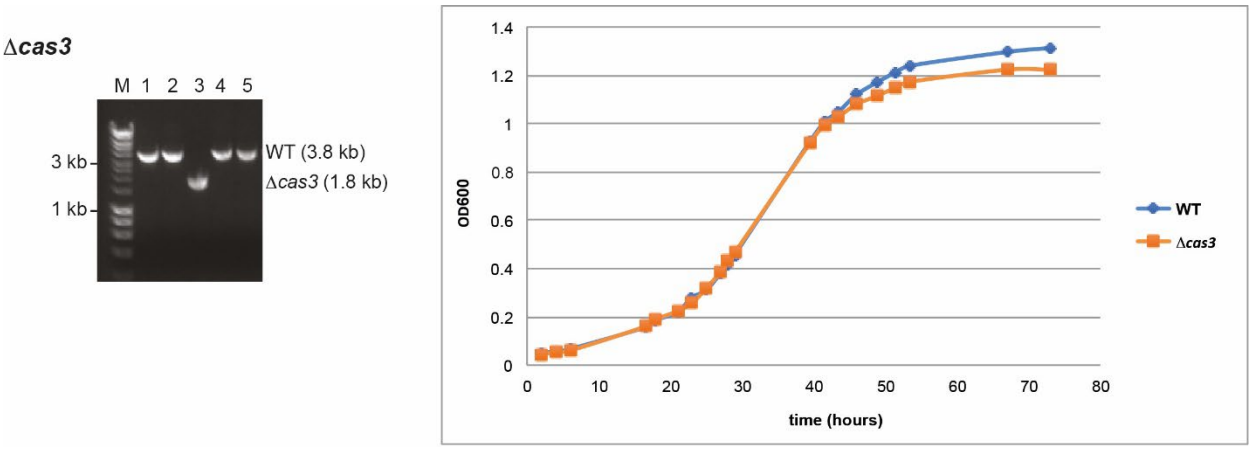


Figure S5: Deletion of *cas3* in *S. acidocaldarius.* A) Deletion of the *cas3* (SACI_RS09010) of *S. acidocaldarius*. **B)** Growth profile (OD600) of MW001 *S. acidocaldarius* cultures (referred to as WT) and Δ*cas3* knockout cultures.

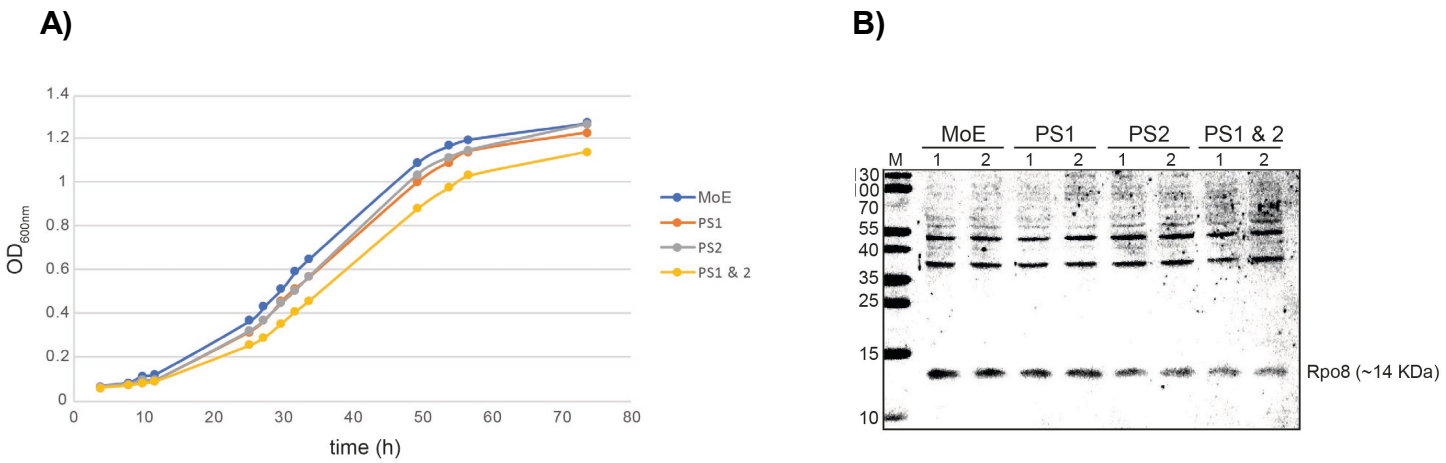


Figure S6: Silencing of *rpo8* using three different miniCR vectors. A) Growth profile (OD600) of control (blue) and three independent *rpo8* silencing cultures transformed with different silencing constructs: miniCR-rpo8-PS1 targeting at PS1 (orange), miniCR-rpo8-PS2 targeting at PS2 (grey) (cf. Fig. 5D main manuscript); multiplex miniCR-rpo8-PS12 targeting at PS1 and PS2 (cf. Fig. 5D main manuscript) (yellow). One biological replicate per transformant shown. **B)** Western blot analyses using Anti-Rpo8 antibodies detecting Rpo8 (indicated). Two biological replicates of control (left) and the individual *rpo8* - silenced cultures (harvested at OD600=0.3) are presented (cf. main manuscript, Fig. 5D). Replicates originated from one grown up colony which has been split into two liquid cultures.

Table S1: Extension to Table 1 in main manuscript. # refers to absolute numbers.

construct	# transformations	# total constructs with TS losses (incl. Gel pics) per trafo	# total sequenced constructs per trafo	# seq-constructs with TS losses per trafo	# TS losses at repeat (of sequenced constructs) per trafo	# constructs with mutations w/o spacer loss per trafo	nature of mutation w/o spacer loss	# constructs with mutations with spacer loss	nature of mutations with spacer loss
CA-1	6	5	6	5	4	1	SNPs in TS, R	3	1x60bp insertion in Leader: 2x SNPs in Leader
CA-3	5	5	5	5	5	0		2	1x60bp insertion in Leader: 1x SNPs all over construct
CA-123	2	2	2	2	2	0		1	1x single bp insertions in leader
aiF5-I	2	1	2	1	1	1	SNPs in TS, SpD1	0	
aiF5-I	3	2	3	2	2	1	SNPS in TS, Leader	0	
SB-123	13	10	2	2	2	N/A	N/A	1	1x 165 bp insertion at R-TS position
SB-23	4	3	2	2	2	N/A	N/A	2	1x 36bp insertion in SpD1 and 129bp insertion in TS; 1x SNPs in SpD1 and SpD5
SB-3x6colonies	5	0	5	0	0	5	SNPs in R position 7 or 9 C--> T	0	
SM2colonies	3	3	3	3	2	0		3	MM and doubling event of SpD5 in c1 anc c3, whole section from leader until SpD5 gone in c2
TOTAL	43	31	30	22	20	8		0	12
% to trafos		65.1%				78.5%			
% to seq trafos					66.7%	62.5%		0.0%	54.5%
% to seq TS loss					90.9%				

Table S2: Primers used in this study.

Spacer-SM2_FW[a]	TAATTTCAGTACAATCTCTTAATACTAAAgataatctcttatagaattga
Spacer-SM2_RV[a]	TAAGAGATTGTACTGAAATTAGGGAAGGTctttcaattctataagagatt
qSM2_FW[b]	AAGGATGGCTCAGAGTACATTGG
qSM2_RV[b]	CCCTACCGTATTTTGCTACTGGT
SM2_FW_Seq[d]	GTGCAAGCAAAAGTAGAAAATCCG
SM2_RV_Seq[d]	TTATTTCTCACTATTCATAACTGTCTCATAGTC
Spacer-CA-1_FW[a]	TAAATGTCTTTAACCTTTTGTCCAATAAAgataatctcttatagaattga
Spacer-CA-1_RV[a]	ACAAAAGGTTAAAGACATTTATGGAAGGGctttcaattctataagagatt
Spacer-CA-2_FW[a]	CTACCTCAATCCCAGTTATACTTCCATCTgataatctcttatagaattga
Spacer-CA-2_RV[a]	GTATAACTGGGATTGAGGTAGCACAAGGTctttcaattctataagagatt
Spacer-CA-3_FW[a]	CATTTTCCGGAATCTCAGATGAGATATAAgataatctcttatagaattga
Spacer-CA-3_RV[a]	CATCTGAGATTCCGGAAAATGCGTACAAActttcaattctataagagatt
qCdvA_FW[b]	TCCTCTAATGCCCTCTGCCTTT
qCdvA_RV[b]	TCATGACCATGGGACCAGAAAG
qCdvB_RV[b]	ACGGAATAGCAGTAGAAACGGGTG
qCdvB_FW[b]	GCTTGTGACGCCCTTGACGA
qCdvC_FW[b]	CACTTTGACATCACTGAGGCAGC
qCdvC_RV[b]	ATGGTCCTCCCGGTTGTGGT
qRS04665_FW[b]	ACACCCCGCAATCCACCCTA
qRS04665_RV[b]	ATACTGACCCCAAAAATCTTCTTGC
qRS0486_FW[b]	GTGTAATTTACGGCTTAACGAAGGC
qRS0486_RV[b]	GATCACGAGGAAAATTGCTATCCC
RS0486-FW_flank[d]	CGAGATTGCCAGAGTAATAAGTGC
RS0486-RV_flank[d]	AAGGGACAAGAGAGGCGGTAG

q16S-FW[b]	TTGGGATCGAGGGCTGAAAC
q16S-RV[b]	CTCACCCCTCTCCTACTCGG
q3194FW[b]	ATCAGTGGAGACGAGTGGCAAGA
q3194RV[b]	ATTGCAGCCTTAACCTCGCCTTCT
Rpo8SACI_PS1_FW[a]	CTATATTCAGGCTTTTCACGTGATATTATgataatctcttatagaattga
Rpo8SACI_PS1_RV[a]	ACGTGAAAAGCCTGAATATAGCGAAAAAGctttcaattctataagagatt
Rpo8_Saci_SP2_FW[a]	TCTTGTTGCCTCTCAAGGAATAGGTAGCCgataatctcttatagaattga
Rpo8_Saci_SP2_RV[a]	ATTCCTTGAGAGGCAACAAGAAGATGGAActttcaattctataagagatt
Saci_1872 Upstream Fw[c]	GCGGccatggAGCTGAGGCGTACTTTGTGATCCCA
Saci_1872 Upstream Rv[c]	ACAAAAGTAGTGCTTTAGGGGACTACCACCTCACGTACATACGT
Saci_1872 Downstream Fw[c]	CGTGAGGTGGTAGTCCCCTAAAGCACTACTTTTGTTTTTATGAG
Saci_1872 Downstream Rv[c]	GCGGggatccTTACACTTTTTAACGCAGTGGACAA
Saci_1872 detect Fw[c]	CAGTTCACCTTAGAAGGTGTACTAATATTT
Saci_1872 detect Rv[c]	ACAGGGTAACCCTAAAACCATTTTACGTAA
saci_0277-Fw[b]	AATATCTTCCATTGAAAAGGGTGCATTAAA
saci_0277-Rv[b]	CCATACAGGGAGATTTCATCTATGAAACTT
saci_1300-Fw[b]	TCCGCAAGGAGGCTTTTCCCCGCTCTAAAA
saci_1300-Rv[b]	GACATTTCACAACACGAGCTGGCGACGGCC
D291FW[e]	ACTATAGCCTTAACGCAGAAGGGT
D291RV[e]	TAGTTGTGTGCCCGCAAAACTG
ORF-904FW[e]	ACAAGAAGAACGGGGGTG
ORF 904RV[e]	ACCTCTTCAGCAATCGCCT
406-FW[e]	ATAAGTTTGATGGGGCAGCA
406-RV[e]	TGACCATGATTACGAATTCGA

[a]primers used for spacer construction (small letters: overlapping repeat regions); [b]primers used in RT-qPCR; [c]primers used to generate Δ*cas3* knockout in *S. acidocaldarius*; [d]primers amplifying the native gene(s) on respective host chromosome (used for qPCR standard generation and sequencing); [e]virus and plasmid – based primers for integrity check and sequencing

Table S3: Protospacer (PS) regions targeted.

Gene and locus tag	**miniCR construct**	**chromosomal locus of PS (c: complement)**
smAP2 SSOP1_RS00995	SM2	c: 174756 – 174792
cdvA SSOP1_RS04680	CA-1	c: 796087 - 796123
	CA-2	c: 796016 – 796052
	CA-3	c: 795656 - 795692
rpo8 Saci_0661	rpo8-PS1	524406 – 524442
	rpo8-PS2	524457 – 524493

Supplementary Results:

Regulatory elements in the *cdvABC* locus (cf. Figure 2A)

When exploring the ORF of *cdvA* (SSOP1_RS04680), a second ATG was identified, situated 84 bp downstream of, and in frame with the annotated start codon. An expression profile drawn from a *S. solfataricus* P1 wild type RNASeq analysis (rRNA depleted, therefore no expression profile for 16S rRNA) (I. Zink, unpublished data) showed a strong increase of the mapped transcripts, which emerged close to the second ATG and proceeded throughout the downstream locus. Complementarily, we found a TATA box located at a specific distance of ~27 bp upstream to this potential transcription start site (TSS). In contrast, transcripts spanning the annotated start codon were underrepresented and no obvious TSS could be identified in our transcriptome data (Fig. 2A), which provides evidence that the second ATG might serve as alternative or the generic translation start. Translation from the second start codon would lead to an N-terminal truncated protein of 239 amino acids (instead of 266) in length, coinciding with the length of the *S. acidocaldarius* CdvA (1).

Further, a TATA box upstream to *cdvB* was found, located within the last 9 bp of *cdvA,* which could potentially serve as a promoter. When screening the relatively short intergenic regions (*cdvA – cdvB:* 18bp, *cdvB – cdvC:* 15bp), we detected a canonical RBS (ribosomal binding site, 5’ GAGGG) 10 bp upstream to *cdvC*, and a non-canonical RBS (5’ GTGAG) located 9 bp upstream to the *cdvB* start codon, respectively (Fig. 2A). In *Sulfolobus and Saccharolobus*, RBSs are predominantly found to proceed ORFs in polycistronic mRNAs allowing for posttranscriptional regulation of gene expression. Further, expression profiles in the intergenic regions did not show a specific decline of read levels, which indicates full transcript coverage

(Fig. 2A, lower panel). Altogether, these observations theoretically would allow both regulatory scenarios of the *cdvABC* locus: *cdvA*-promoter-mediated expression of a polycistronic transcript consisting of all three ORFs with RBSs allowing for posttranscriptional regulation of CdvBC proteins; or *cdvA*-independent transcription of the *cdvB-cdvC* unit into a bicistronic mRNA initiated by the *cdvB* promoter, indicative for regulation on the transcriptional level.

References:

1) Samson RY, Obita T, Hodgson B, Shaw MK, Chong PLG, Williams RL, Bell SD. 2011. Molecular and Structural Basis of ESCRT-III Recruitment to Membranes during Archaeal Cell Division. Mol Cell 41:186–196.