

SUPPLEMENTARY MATERIAL

Selection of tetracycline inducible self-cleaving ribozymes as synthetic devices for gene regulation in yeast

Alexander Wittmann and Beatrix Suess

Institute for Molecular Biosciences, Goethe Universität Frankfurt am Main, Max-von-Laue
Straße 9, 60438 Frankfurt am Main, Germany

*Corresponding author: suess@bio.uni-frankfurt.de

Phone: +49 69 798 29785

Fax: +49 69 798 29323

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Table S1: Sequences of the randomized linker regions of 75 ribozymes.

construct	R1	R2	R3	construct	R1	R2	R3
9.1	AAUGG	CCAUC	AUUA	9.39	AUUGG	n.d.	AAAA
9.2	GAGG-	CCUGG	GUCU	9.40	CUUC-	GAAUG	ACCC
9.3	ACCGG	ACUCA	CGAA	9.41	UAGG-	CCUGC	CUCC
9.4	GCCCU	AGGAG	UUAC	9.42	AAACC	UCAUU	UAUU
9.5	AGCGG	CCGCC	ACAA	9.43	AAUGG	CCUUC	UAA-
9.6	AAGGU	ACCCA	ACUU	9.44	CACA-	UGUGA	UUUA
9.7	GAUCU	AGGCG	CUCC	9.45	CGUGA	UCCUU	ACUU
9.8	AUUGG	n.d.	AAAA	9.46	GGCCU	AGGAG	CUCC
9.9	AGAGG	CCUCA	AAAA	9.47	ACUGG	n.d.	AAA-
9.10	AAGGG	n.d.	GCCG	9.48	AACGG	CCGCU	CUCA
9.11	AUAGG	CCUCG	AUUA	9.49	CAGGA	UUCUG	ACCU
9.12	ACGGA	UCCCA	CUU-	9.50	AUGGA	UCCCA	ACU-
9.13	ACCGG	ACUCA	CGAA	11.1	n.d.	n.d.	n.d.
9.14	CGUGA	UCUGG	UAAU	11.2	ACUAG	n.d.	AAAA
9.15	GUCCA	UGGCA	ACU-	11.3	AUAGG	AAGC-	UACC
9.16	GUAC-	GUCUG	ACU-	11.4	AGCCU	AGGAG	AUCU
9.17	GAG--	AUUCA	GAAA	11.5	AAUGG	CCAUC	AAUA
9.18	AAGGU	ACCCA	ACUU	11.6	ACUAG	n.d.	ACCG
9.19	UGUCC	GGUG-	CUCC	11.7	UACCG	CGGUC	CUCC
9.20	ACACU	AGUUU	AUAA	11.8	AUCGG	n.d.	AAAA
9.21	GGCCU	AGGAG	CUCC	11.9	UAGGA	UCCCA	CUUA
9.22	AAUGG	CCAUC	ACUU	11.10	GUGAU	AUCCA	CUUU
9.23	AGUGC	GCCCU	CUUU	11.11	CUCGG	n.d.	AAAA
9.24	AGGCC	GACCA	AAUA	11.12	n.d.	n.d.	n.d.
9.25	CGGU-	UGGCU	UACA	11.13	ACUAG	n.d.	A---
9.26	GUCCG	CGGCG	AACC	11.14	n.d.	n.d.	n.d.
9.27	GUCCA	n.d.	AAG-	11.15	ACCAG	AUUGC	CUCC
9.28	GACAG	n.d.	AAA-	11.16	AUCGA	UCGCA	AAUA
9.29	AUCGG	CCGUC	UCGU	11.17	AUAGG	n.d.	AAA-
9.30	AGAAG	n.d.	AAAA	11.18	GAUGG	UCAUC	ACCU
9.31	ACUGG	CCAUC	CGUA	11.19	AACGG	CCGCA	AACU
9.32	UUUGU	n.d.	AAA-	11.20	UCGGA	UCCCA	ACUU
9.33	UCCA-	UGGCG	UCUC	11.21	AAGGA	UCCU-	CUCC
9.34	AAGGA	UCCCA	CGUA	11.22	CGGG-	CCCUC	CUCC
9.35	UUGG-	CCUCG	CACC	11.23	AGCAG	n.d.	A---
9.36	UGCUU	GAGCG	ACCC	11.24	UCCGG	GAUCC	AAAA
9.37	AGAAU	AUUCC	ACGA	11.25	CGGU-	ACCUC	CUUC
9.38	UCGGC	GCUUC	AUCG				

Presented are the sequences of R1, R2 and R3 of 75 ribozymes isolated from selection cycle C9 and C11 in 5' to 3' orientation (see Figure 1). In some constructs, R2 or R3 was deleted (marked with n.d.).

Table S2: Statistic analysis of the pool constructs of selection cycle C9 and C11.

cycle C9	cr	R1	R2	R3
sequences [N]	50			
length [nt]	114	5	5	4
nt's total	5700	250	250	200
pm [N]	115	-	-	-
pm [%]	2,0	-	-	-
snd [N]	150	8	31	9
snd [%]	2,6	3,2	12	4,5
alterations total [%]	4,6	6,9		

cycle C11	cr	R1	R2	R3
sequences [N]	25			
length [nt]	114	5	5	4
nt's total	2850	125	125	100
pm [N]	91	-	-	-
pm [%]	3,2	-	-	-
snd [N]	273	21	40	18
snd [%]	9,6	17	32,0	18,0
alterations total [%]	12,8	22,6		

Specified are nucleotide alterations within the constant region (cr) and the randomized regions (R1, R2, R3) of the pool construct of selection cycle C9 and C11. Analysed were point mutations (pm) and single nucleotide deletions (snd).

Table S3: Detailed analysis of several ribozymes.

	sequence			base pairing		k _{obs}		dynamic range	group	comment
	R1	R2	R3	UGGA/R3	R1/R2	-tc	1 μM tc			
9.1	AAUGG	CCAUC	AUUA	XXXO	OXXXX	0,025	0,766	30,2	III	
9.3	ACCGG	ACUCA	CGAA	XOOO	OXXOXO	nc			IV	
9.6	AAGGU	ACCCA	ACUU	OXXO	OXXXX	0,009	0,414	45,5	II	
9.7	GAUCU	AGGCG	CUCC	OXXO	OXXXX	nc			I	C39U
9.8	AUUGG	n.d.	AAAA	n.d.	n.d.	nc			V	
9.9	AGAGG	CCUCA	AAAA	XOOO	OXXXX	0,020	0,604	30,9	VII	
9.11	AUAGG	CCUCG	AUUA	XXXO	OXXXX	0,031	0,755	24,4	VII	
9.12	ACGGA	UCCCA	CUU-	OXXD	OXXXX	0,009	0,848	92,5	VII	
9.13	ACCGG	ACUCA	CGAA	XOOO	OXXOXO	nc			IV	
9.18	AAGGU	ACCCA	ACUU	OXXO	OXXXX	0,010	0,428	43,2	II	
9.19	UGUCC	GGUG-	CUCC	OXXO	XXDXX	0,017	1,126	64,7	I	
9.21	GGCCU	AGGAG	CUCC	OXXO	OXXXX	0,023	0,969	41,4	I	
9.22	AAUGG	CCAUC	ACUU	OXXO	OXXXX	0,075	1,381	18,5	II/III	
9.23	AGUGC	GCCCU	CUUU	OXXO	XXOXX	0,026	0,891	34,4	VII	
9.35	UUGG-	CCUCG	CACC	OXOO	XODXX	uc			VII	
9.41	UAGG-	CCUGC	CUCC	OXXO	DXXXX	0,036	1,289	35,4	I	
9.43	AAUGG	CCUUC	UAA-	XDOX	OXOXX	0,006	0,429	68,1	III	
9.46	GGCCU	AGGAG	CUCC	OXXO	OXXXX	0,031	1,062	33,9	I	
9.50	AUGGA	UCCCA	ACU-	DXXO	OXXXX	0,005	0,449	96,2	II	
11.4	AGCCU	AGGAG	AUCU	OXXO	OXXXX	0,002	0,666	332,8	VII	
11.5	AAUGG	CCAUC	AAUA	XXOO	OXXXX	0,022	0,388	18,0	III	
11.7	UACCG	CGGUC	CUCC	OXXO	OXXXX	uc			I	U41C
11.10	GUGAU	AUCCA	CUUU	OXXO	OXXXX	0,008	0,751	91,5	VII	
11.18	GAUGG	UCAUC	ACCU	OXXO	XXXXX	0,025	1,129	45,4	VII	
11.20	UCGGA	UCCCA	ACUU	OXXO	XOXXX	0,009	0,732	79,5	II	
11.21	AAGGA	UCCU-	CUCC	OXXO	DXXXX	0,013	0,337	26,1	I	
11.22	CGGG-	CCCUC	CUCC	OXXO	DOXXX	0,007	0,644	91,3	I	
11.25	CGGU-	ACCUC	CUUC	OXXO	DOXXX	0,021	0,706	34,3	VII	

Presented are the observed cleavage rate constants in absence and in presence of 1 μM tetracycline of 28 ribozymes and the calculated dynamic ranges (k_{obs}^+/k_{obs}^-). In addition, the sequences and potential base pairing patterns of R1, R2 and R3 are specified. Sequences are shown in 5' to 3' orientation and base pairing patterns are shown in 5' to 3' orientation of UGGA and R1 (see Fig.1). Watson-Crick and wobble base pairing (G-C, A-U and G-U) is indicated by "X", no base pairing is indicated by "O". Constructs that cleaved even in absence of tetracycline are labeled with "uc" (uninduced cleavage); constructs that did not cleave at all are labeled with "nc" (no cleavage).

Figure S1: Nucleotide distribution in R1, R2 and R3.

Shown is the nucleotide distribution in R1, R2 and R3 of 50 sequences of selection cycle C9, 25 sequences of cycle C11, all 75 sequences of cycle C9 and C11 and 22 tetracycline inducible hammerhead ribozymes. Frequency plots were generated using WebLogo (<http://weblogo.berkeley.edu/>).



Figure S2: Gel electrophoreses of 4 allosteric ribozymes.

Depicted are ribozymes 9.12, 9.19, 9.50 and 11.22 analysed by gel electrophoreses. RNAs were incubated under same buffer conditions as in cleavage kinetics. Increasing volumes of the corresponding master mixes were loaded on the gels. Electrophoreses were performed under A) denaturing and B) native conditions.

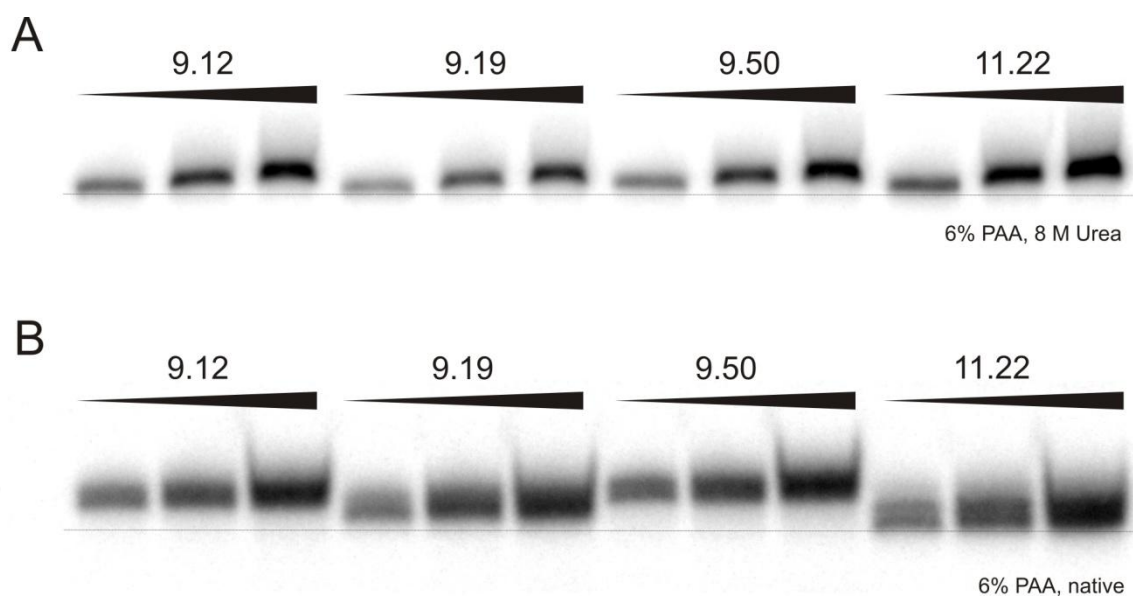


Figure S3: Sequences and secondary structures of the ribozyme controls used for yeast and mammalian cell culture reporter gene assays.

A) The parental hammerhead ribozyme N79 and a catalytically inactivated N79 (A64G) were used as positive and negative control (“hh” and “hhi”). B) To test whether the fusion of ribozyme and aptamer has general impact on ribozyme activity, we generated the design control “dc”. The linker sequence permits perfectly structured UGGA/R3 and R1/R2 stems and should therefore enable hammerhead cleavage. C) Shown are mutants of 9.19 where either ribozyme cleavage or tetracycline binding is inhibited. The black arrows mark the hammerhead cleavage site.

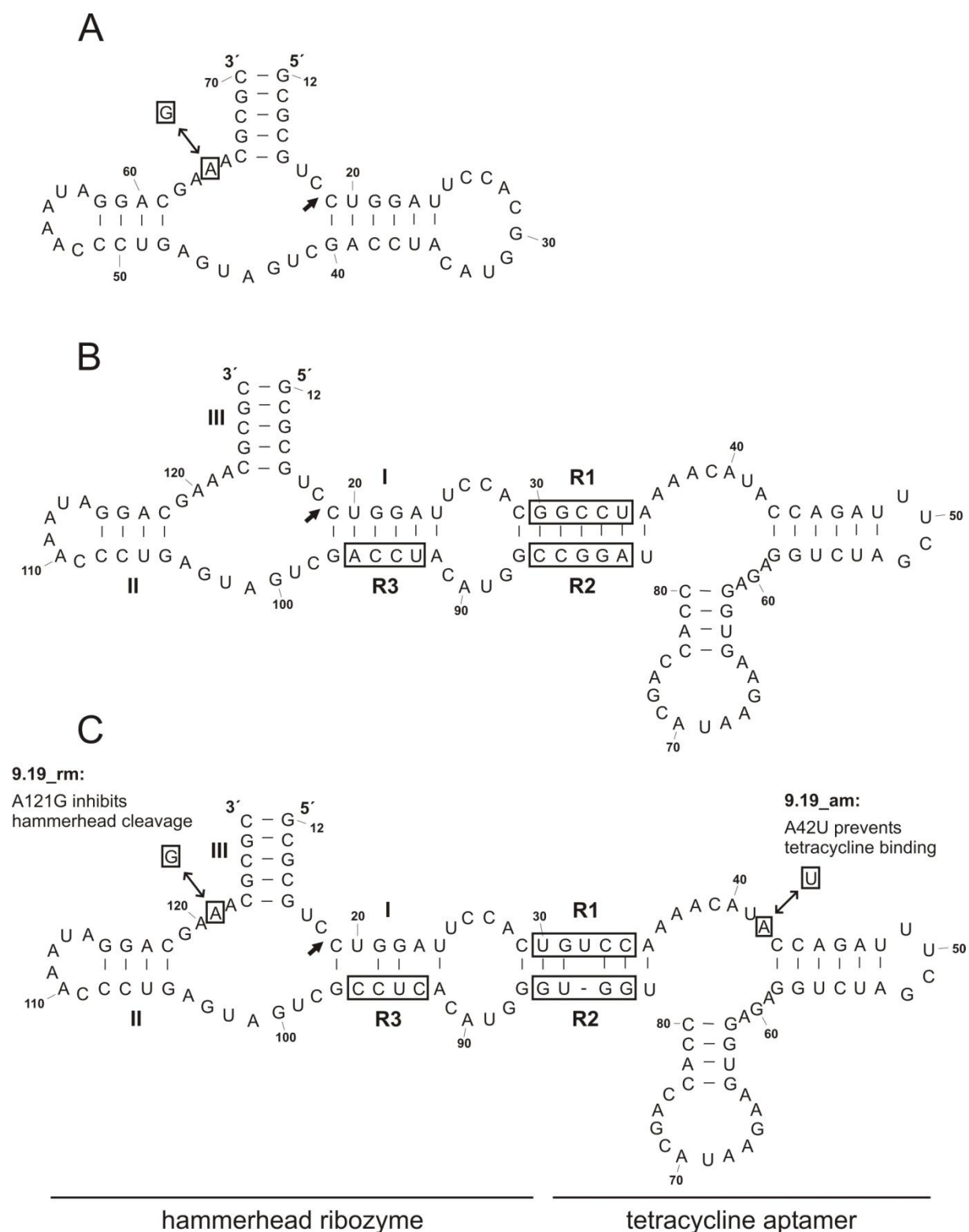
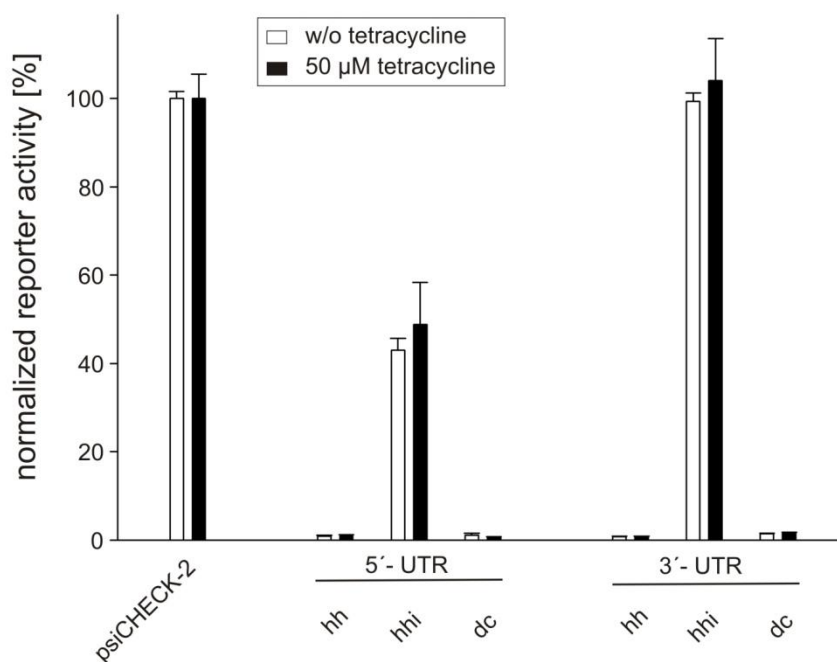


Figure S4: Mammalian cell culture reporter gene assay.

Ribozyme controls were tested in HeLa cells. Depicted is the normalized reporter gene activity of psiCHECK-2 and derivatives containing ribozyme controls “hh”, “hhi” or “dc” in the 5' or 3'UTR of renilla luciferase. The controls “hh” and “hhi” represent functional active and inactive hammerhead ribozymes, respectively. The design control “dc” comprises a hammerhead-aptamer fusion based on our pool construct with linker sequences that facilitate hammerhead cleavage to check whether the aptamer has an impact on hammerhead cleavage (Supplementary Figure S3A,B). White and black bars represent the normalized reporter gene activity in absence and presence of 50 μ M tetracycline, respectively. Shown are mean values and standard deviations of three independent measurements.



Supplementary methods

Plasmid construction, mammalian cell culture and luciferase reporter gene assay

The vector psiCHECK-2 (Promega), which constitutively expresses renilla and firefly luciferase from a SV40 early and HSV-TK promoter, respectively, was used for ribozyme cleavage assays in mammalian cells. Ribozyme variants were cloned into the 5' and 3' UTR of renilla luciferase in close proximity to the start or stop codon using either the unique restriction sites NheI (5' UTR) or XhoI and NotI (3' UTR). All ribozymes that were inserted into the 5' UTR contained the point mutation U102C (see Figure 1) to remove an additional start codon. The parental ribozyme N79 (termed "hh") and a corresponding inactive variant carrying a point mutation in the catalytic core (termed "hhi") were used as positive and negative controls (Supplementary Figure S3A). As design control, we cloned "hh" with a fused tetracycline aptamer via linker sequences that permit perfectly structured linker stems (termed "dc", R1: 5'-GGCCU-3', R2: 5'-AGGCC-3', R3: 5'-UCCA-3', see Supplementary Figure S3B). Ribozymes were flanked with CA-rich spacer elements.

HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum superior (FCS) (Biochrom AG), 100 µg/ml streptomycin (PAA), 100 U/ml penicillin (PAA) and 2 mM L-glutamine (PAA) at 37°C with 5% CO₂. One day before transfection, cells were seeded in 96-well plates at a density of 1.5×10^4 cells per well. The plasmids were transiently transfected using 50 ng of plasmid and 0.5 µl lipofectamine-2000 (Invitrogen) per well according to the manufacturer's instructions. The transfection medium was removed two hours after transfection and cells were incubated for 24 hours in cell culture medium mentioned above supplemented with 50 µM tetracycline.

Luminescence measurements were performed using the Dual Glow[®] Luciferase Assay System from Promega according to the manufacturer's instructions. Luciferase light emission was monitored with a Tecan Infinite M200 luminometer using one second integration time. The ratio between Renilla and Firefly luciferase was calculated for each well to normalize for varying transfection efficiencies. Mean values and standard deviations were calculated from triplicate samples and normalized to the value of the vector without ribozyme insertion. Each experiment was repeated at least two times.

Supplementary Figure S4 shows the normalized renilla luciferase expression levels of the controls "hh", "hhi" and "dc". The inactive hammerhead ribozyme ("hhi") itself did not interfere with gene expression when inserted into the 3' UTR but reduced reporter gene activity to ~ 50% when cloned into the 5' UTR. In contrast, the active hammerhead ribozyme ("hh") led to strong reduction of gene expression in both 5' and 3' UTR, demonstrating the great potential of self-cleaving ribozymes for conditional gene regulation. Addition of 50 µM tetracycline did not inhibit full-length hammerhead cleavage in HeLa cells. Additionally, the ribozyme with attached tetracycline aptamer ("dc") showed a similar effect as the ribozyme alone indicating that the aptamer domain itself does not interfere with ribozyme cleavage in our construct design. Unfortunately, none of our tested constructs showed significant reduction of reporter gene activity in presence of 50 µM tetracycline, neither when integrated in the 5' nor the 3' UTR (data not shown).