SUPPLEMENTARY DATA

Folding a Stable RNA Pseudoknot through Rearrangement of Two Hairpin Structures

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Table S1. Quantification of unfolding transitions

Construct	Wild type				
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip of HR	Rip of BR	Rip of BR[2]
Structural change	mixed	H1 to SS	H1 to SS	PK to SS	PK to SS
# of nt released[a]	mixed	27-29 nt	27-29 nt	67 nt	67 nt
N (rips)	810	826	649	258	232
unfolding force (pN)	11.0 ± 1.7	14.3 ± 0.8	14.3 ± 0.7	18.0 ± 2.3	15.1 ± 1.4
extension change (nm)	10.0 ± 2.2	9.9 ± 1.4	9.8 ± 1.2	24.9 ± 1.4	23.9 ± 1.2
# of nt change, calibrated[b]	N/A[e]	28.3 ± 3.4	28.1 ± 2.8	66.8 ± 2.7	66.6 ± 2.4
work under rip (KJ/mol)[c]	66.2 ± 22.6	83.4 ± 11.5	82.5 ± 9.9	260.5 ± 42.7	210.5 ± 26.3
ΔG (KJ/mol)[d]	N/A[e]	38.3 ± 2.2	40.6 ± 1.3	124.0 ± 5.2	110.5 ± 3.4

Construct	mHP	
Transition	Rip 1 of 2R	Rip 2 of 2R
Structural change	H2 to SS	H1 to SS
# of nt released[a]	26-30 nt	27-29 nt
N (rips)	291	292
unfolding force (pN)	9.8 ± 1.6	13.9 ± 0.6
contour-length change (nm)	8.9 ± 1.9	9.7 ± 1.0
# of nt change, calibrated[b]	28.7 ± 4.2	28.0 ± 2.4
work under rip (KJ/mol)[c]	52.8 ± 18.7	79.6 ± 8.5
ΔG (KJ/mol)[d]	14.9 ± 1.0	42.0 ± 1.4

Construct	mS1L		mS2L	
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip 1 of 2R	Rip 2 of 2R
Structural change	H2 to SS	H1 to SS	H2 to SS	H1 to SS
# of nt released[a]	26-30 nt	27-29 nt	26-30 nt	27-29 nt
N (rips)	220	246	578	577
unfolding force (pN)	9.0 ± 0.6	16.3 ± 0.5	12.2 ± 0.7	14.0 ± 0.7
extension change (nm)	8.3 ± 0.9	9.5 ± 0.7	7.9 ± 1.0	9.8 ± 1.1
# of nt change, calibrated[b]	27.8 ± 2.3	26.6 ± 1.7	24.5 ± 2.6	28.1 ± 2.6
work under rip (KJ/mol)[c]	44.3 ± 6.5	91.8 ± 6.8	56.8 ± 7.9	81.0 ± 8.6
ΔG (KJ/mol)[d]	19.8 ± 1.3	53.7 ± 2.0	28.1 ± 0.7	38.8 ± 1.9

Construct	mPK			
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip of BR	Rip of BR[2]
Structural change	mixed	H1 to SS	PK to SS	PK to SS
# of nt released[a]	mixed	27-29 nt	67 nt	67 nt
N (rips)	522	614	11	29
unfolding force (pN)	9.0 ± 1.8	13.9 ± 0.6	18.4 ± 4.4	14.1 ± 1.0
extension change (nm)	8.9 ± 2.3	9.5 ± 1.0	24.6 ± 1.5	23.3 ± 0.8
# of nt change, calibrated[b]	N/A[e]	27.6 ± 2.5	66.0 ± 2.0	66.2 ± 1.5
work under rip (KJ/mol)[c]	49.2 ± 21.3	78.2 ± 7.9	264.1 ± 76.3	192.9 ± 17.4
ΔG (KJ/mol)[d]	N/A[e]	38.3 ± 3.0	129.5 ± 17.5	113.7 ± 3.6

Construct	mPK2			
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip of BR	Rip of BR[2]
Structural change	mixed	H1 to SS	PK to SS	PK to SS
# of nt released[a]	mixed	27-29 nt	67 nt	67 nt
N (rips)	761	760	23	49
unfolding force (pN)	9.5 ± 1.8	13.9 ± 0.6	18.3 ± 4.1	14.3 ± 1.3
extension change (nm)	10.6 ± 1.4	10.3 ± 0.9	24.9 ± 1.5	23.6 ± 1.0
# of nt change, calibrated[b]	N/A[e]	29.4 ± 2.3	66.7 ± 1.6	66.8 ± 1.9
work under rip (KJ/mol)[c]	59.7 ± 18.4	84.7 ± 7.5	265.3 ± 76.7	196.7 ± 22.5
ΔG (KJ/mol)[d]	N/A[e]	47.4 ± 0.7	124.0 ± 11.9	105.4 ± 5.6

Construct	mAC				mAC2
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip of BR	Rip of BR[2]	Rip
Structural change	mixed	H1 to SS	PK to SS	PK to SS	H1 to SS
# of nt released[a]	mixed	27-29 nt	67 nt	67 nt	27-29 nt
N (rips)	1104	1104	100	92	329
unfolding force (pN)	9.1 ± 2.3	14.2 ± 0.8	17.7 ± 2.9	15.1 ± 1.8	14.3 ± 0.8
extension change (nm)	7.7 ± 2.2	9.6 ± 1.0	23.6 ± 1.5	22.8 ± 1.3	9.1 ± 0.9
# of nt change, calibrated[b]	N/A[e]	27.7 ± 2.4	63.9 ± 2.4	64.1 ± 2.3	26.5 ± 2.4
work under rip (KJ/mol)[c]	43.9 ± 22.8	80.6 ± 8.7	243.0 ± 52.9	201.6 ± 34.5	77.1 ± 7.8
ΔG (KJ/mol)[d]	N/A[e]	38.8 ± 5.3	109.1 ± 9.5	104.8 ± 8.8	41.9 ± 0.9

Construct	mACd14			
Transition	Rip 1 of 2R	Rip 2 of 2R	Rip of BR	Rip of BR[2]
Structural change	PK to H1	H1 to SS	PK to SS	PK to SS
# of nt released[a]	24-26 nt	27-29 nt	53 nt	53 nt
N (rips)	273	273	423	216
unfolding force (pN)	13.1 ± 1.2	14.3 ± 1.0	18.2 ± 2.1	15.4 ± 1.8
extension change (nm)	7.1 ± 1.2	9.5 ± 1.0	18.6 ± 1.1	18.0 ± 1.1
# of nt change, calibrated[b]	23.0 ± 2.6	27.4 ± 2.3	52.2 ± 1.9	52.5 ± 2.2
work under rip (KJ/mol)[c]	55.3 ± 12.5	80.0 ± 10.1	198.4 ± 31.8	163.3 ± 24.6
ΔG (KJ/mol)[d]	22.7 ± 1.0	41.0 ± 2.9	94.2 ± 4.0	81.7 ± 1.8

Data are presented as mean ± SD.

Abbreviations: nt, nucleotide; H1, Hairpin 1; H2, Hairpin 2; SS, single strand; PK, pseudoknot.

[a]Expected numbers of nucleotides released from the designed structural change. A range is given for those involving Hairpin 1 or 2, because the weak G:U or A:U closing base pairs in the hairpins may or may not pair under force.

[b]Calibrated by the worm-like chain model for the designated structural change.

[c]The area under the rip in the force-extension curve. The calculated work includes an energy input to tether (stretch) the released ssRNA strand (33).

[d]Free energy change for the designated structural change. Unlike hopping, the rip transitions listed in the Table are irreversible processes. Thus, we calculated the free energy change by Jarzynski's equality (51). The RNA tethering energy has been subtracted.

[e]Not applicable; including a variety of structural transitions.

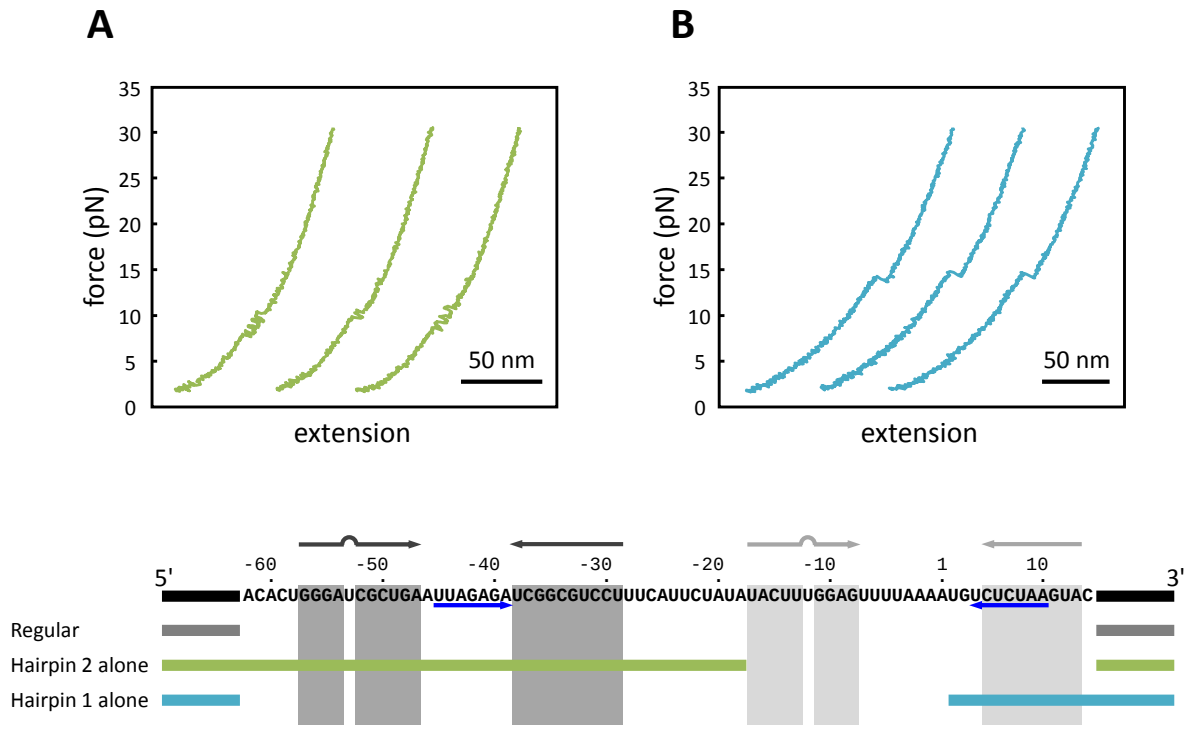

Figure S1. Representative force-extension curves for the wild-type RPSOutr constructs with Hairpin 2 alone **(A)** and Hairpin 1 alone **(B)**. The constructs containing individual hairpins were made by extending either the 5' or 3' single-stranded DNA handle to block formation of the other hairpin, as demonstrated in the bottom panel. The hopping (8–10 pN) or rip (at approximately 15 pN) appeared as the only transition for the construct of Hairpin 2 alone (A) or Hairpin 1 alone (B), respectively.

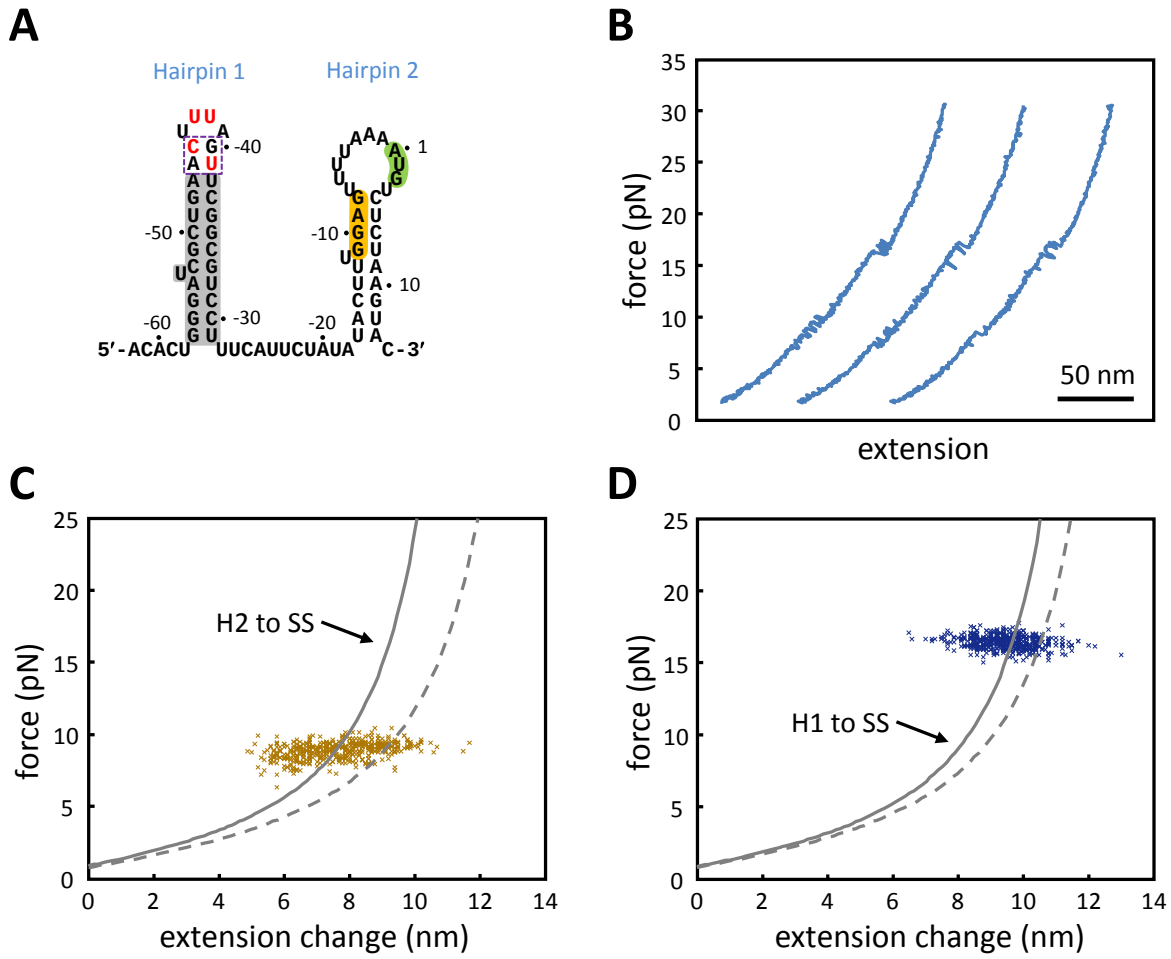

Figure S2. Characterization of the mS1L mutant. **(A)** The predicted structure from *mfold*. Mutated nucleotides are shown in red. Compared to the wild type, Hairpin 1 in mS1L was further stabilized by two extra base pairs. **(B)** Representative force-extension curves. The hopping at 7–10 pN corresponded to Hairpin 2, as in the wild type, with an unfolding free energy of 19.8 ± 1.3 KJ/mol (Supplementary Table S1). The hopping/rips at approximately 17 pN corresponded to the mutated Hairpin 1, with an unfolding free energy of 53.7 ± 2.0 KJ/mol (Supplementary Table S1). Compared to the wild type (38.3 ± 2.2 KJ/mol), the mutated hairpin was further stabilized by -15.4 KJ/mol. **(C)** Distribution of the force and extension change for Hairpin 2 unfolding (N = 507). Two worm-like chain (WLC) models for the unfolding of stem-loops with a total of 26 nt (solid line) and 30 nt (dashed line) are shown. The number of nucleotides (nt) in this hairpin is expected to be 30 (see panel **A**). However, the two weak A:U base pairs in the helix/single-strand junction were usually not detectable under force measurements, and thus the transitions matched better to the model of the hairpin with 26 nt (solid line). **(D)** Distribution of the force and extension change for Hairpin 1 unfolding (N = 620). Two WLC models for the unfolding of stem-loops with a total of 27 nt

(solid line) and 29 nt (dashed line) are shown. The number of nucleotides in this hairpin is expected to be 29 (see panel **A**). Because of the weak G:U base pair in the junction, the measured transitions were matched better to the model of the hairpin with 27 nt (solid line). H1, Hairpin 1; H2, Hairpin 2; SS, single strand.

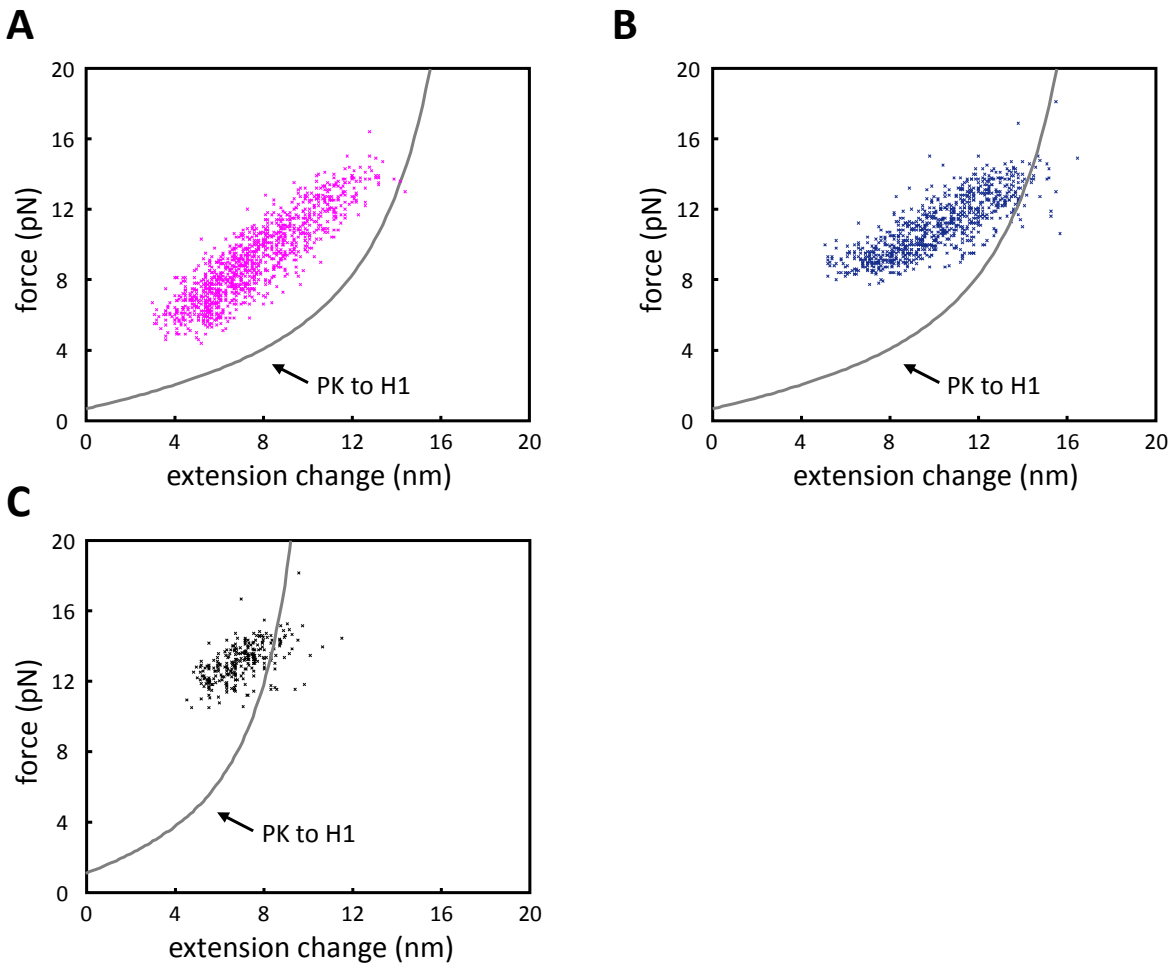

Figure S3. Distributions of the first-rip transitions of 2R patterns. Data from mAC (Panel **A**; N = 1146), the wild type (Panel **B**; N = 832; same as in Figure 2D), and mACd14 (Panel **C**; N = 273). The WLC models corresponding to the transition from the pseudoknot (PK) to Hairpin 1 (H1) are plotted in each panel.

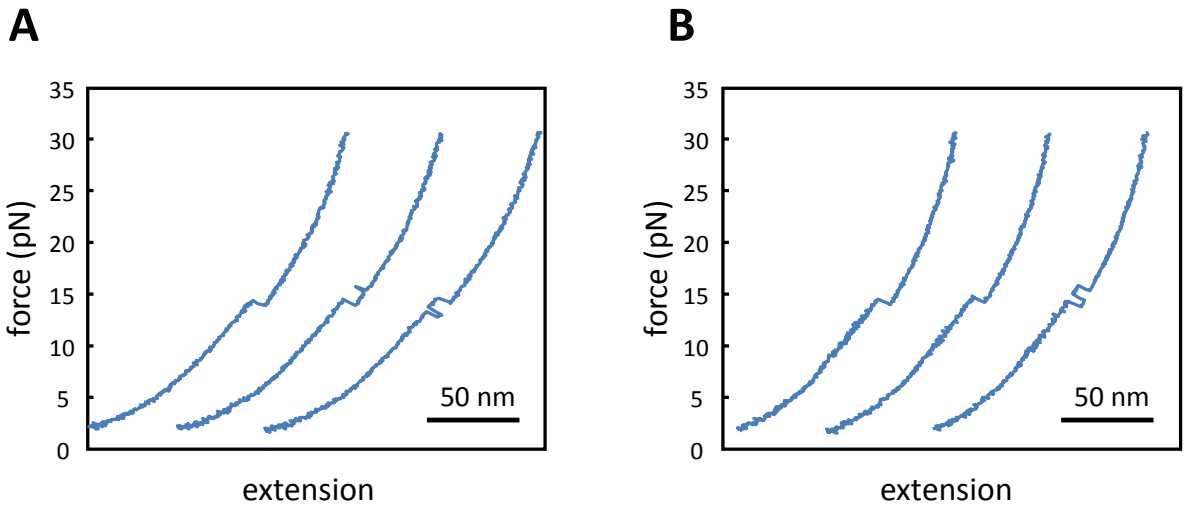

Figure S4. Representative force-extension curves. **(A)** Data from the modified mAC construct, the 3' handle of which was extended (as in Supplementary Figure S1B) to disrupt the base-pairing for Hairpin 2. **(B)** Data from mAC2 with regular handles. In both cases, the only transitions at approximately 15 pN were corresponding to the unfolding of Hairpin 1. Note that the unfolding of this hairpin usually occurred in a single step (rip), but limited cycles of unfolding-refolding (slow hopping) were also observed, as shown in the last traces of both panels.

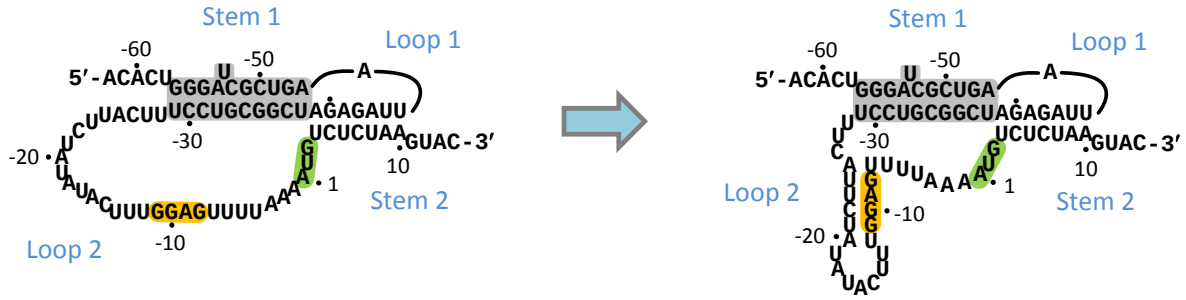

Figure S5. Loop 2 of the wild-type pseudoknot may potentially fold into a small stem-loop structure (predicted by *mfold*). The 6-bp stem contains only one G:C base pair and is marginally stable (ΔG = -10.9 KJ/mol). A slightly different stem-loop fold was proposed previously (23,24).

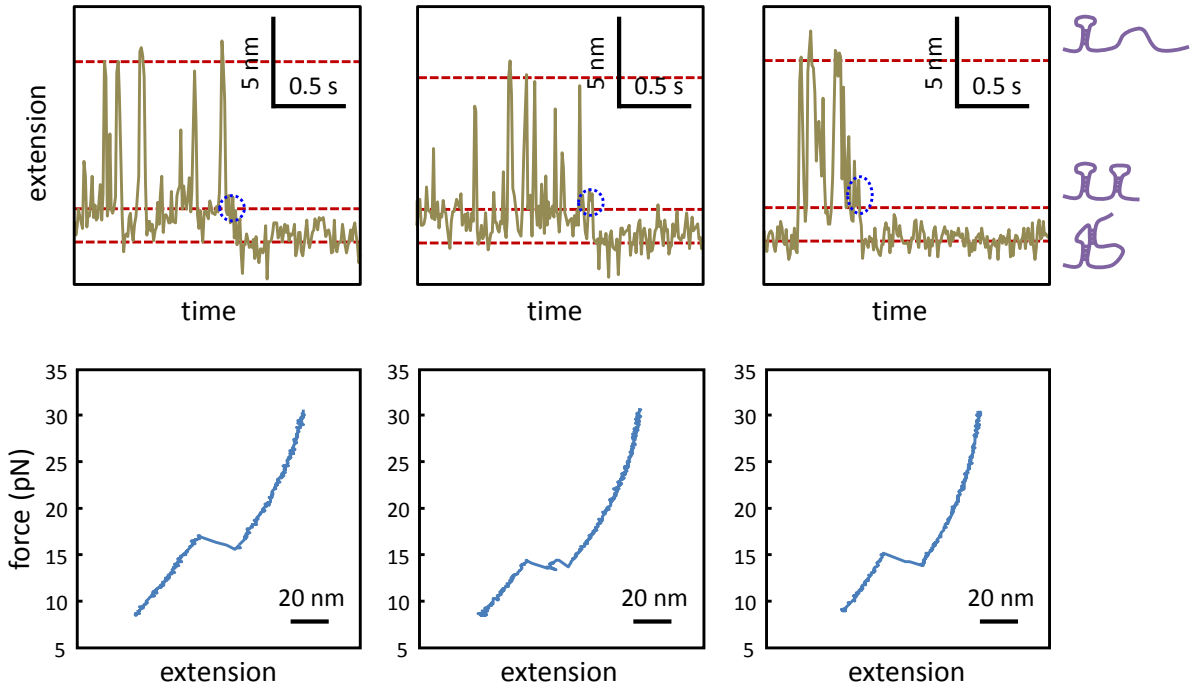

Figure S6. Examples from constant-force experiments. Shown are time-evolved extension change (top panels) of RPSOutr under a pre-set force (8.5, 8.5 and 9 pN from left to right). RNA conformations corresponding to each extension state (dashed lines) are illustrated to the right. Possible folding intermediates leading to the pseudoknot are indicated by blue dotted circles. The follow-up force-extension curves from each example are shown at the bottom.

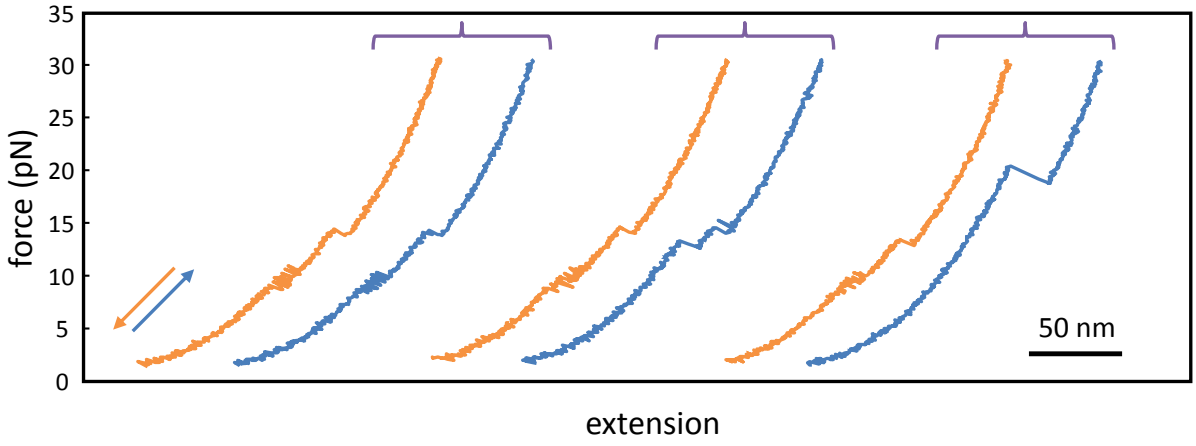

Figure S7. Representative force-extension curves of consecutive refolding-unfolding cycles. RPSOutr was repeatedly pulled (to unfold the structures) and relaxed (to allow structures to refold) many times between 2 and 30 pN. Shown are three cycles of refolding (brown traces) followed by unfolding (blue traces) processes. Each pair of curves from each cycle was shifted horizontally for better visualization. As shown in the figure, all the refolding patterns (from high to low forces) were very similar, with a "zip" (closure of a structure, most likely Hairpin 1) at approximately 14 pN followed by hopping at 8 – 10 pN (closure of a bistable hairpin, most likely Hairpin 2). Thus, the refolding trace is like the reversal of the HR pattern, indicating that the double-hairpin conformation was the first structure to form during the refolding process. However, different types of the unfolding patterns (from left to right: HR, 2R and BR) can appear in the following unfolding process, suggesting that structural rearrangement from the double hairpin to pseudoknot can occur at low forces.

SUPPLEMENTARY REFERENCES

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