Structure-based insights into self-cleavage by a four-way junctional twister-sister ribozyme

Luqian Zheng1, Elisabeth Mairhofer2, Marianna Teplova3, Ye Zhang1, Jinbiao Ma4,5, Dinshaw J. Patel3, Ronald Micura2 & Aiming Ren1,5

Here we report on the crystal structure and cleavage assays of a four-way junctional twister-sister self-cleaving ribozyme. Notably, 11 conserved spatially separated loop nucleotides are brought into close proximity at the ribozyme core through long-range interactions mediated by hydrated Mg2+ cations. The C62–A63 step at the cleavage site adopts a splayed-apart orientation, with flexible C62 directed outwards, whereas A63 is directed inwards and anchored by stacking and hydrogen-bonding interactions. Structure-guided studies of key base, sugar, and phosphate mutations in the twister-sister ribozyme, suggest contributions to the cleavage chemistry from interactions between a guanine at the active site and the non-bridging oxygen of the scissile phosphate, a feature found previously also for the related twister ribozyme. Our four-way junctional pre-catalytic structure differs significantly in the alignment at the cleavage step (splayed-apart vs. base-stacked) and surrounding residues and hydrated Mg2+ ions relative to a reported three-way junctional pre-catalytic structure of the twister-sister ribozyme.
There has been considerable interest towards an improved understanding of the cleavage mechanisms that underlie RNA catalysis by small self-cleaving ribozymes involved in self-scission during rolling circle replication of viral genomes. Such challenges are highlighted by the recent identification of a new set of self-cleaving ribozymes from genomic bioinformatics searches, named twister, twister-sister, pistol, and hatchet ribozymes. These nucleolytic ribozymes site-specifically cleave phosphodiester linkages by activating the 2'-OH of the ribose 5'- to the scissile phosphate for inline attack on the adjacent P-O5' bond to yield 2',3'-cyclic phosphate and 5'-OH ends. The key mechanistic issues relate to the geometry of the adjacent P-O5' alignment at the cleavage site with respect to the attacking 2'-O-P-O5' alignment and the presence/absence of hydrated Mg2+ cations at the scissile phosphate. All structures are in agreement with the conclusion that the pre-catalytic fold of the twister ribozyme provides conformational flexibility of the pyrimidine nucleoside at the pyrimidine–purine cleavage step in contrast to the anchored purine nucleoside, suggestive of a likely realignment of the pyrimidine and its 2'-OH to achieve inline alignment in the transition state. Interestingly, this ribozyme can cleave a single nucleoside (5' of the scissile phosphate) with only twofold reduced rate and despite the inability to form the phylogenetically

Fig. 1 Schematic and tertiary structure of the twister-sister ribozyme. a Schematic of the secondary fold of the dC62-containing four-way junctional twister-sister ribozyme. The sequence is color-coded according to helical segments observed in the tertiary structure. All highly conserved residues are labeled by red circles. Long-distance interactions observed in the tertiary structure are labeled with dashed lines named T1 (in red) and T2 (in black). The cleavage site step was shown by a red arrow. b Schematic of the tertiary fold based on the crystal structure of the dC62-containing four-way junctional twister-sister ribozyme color-coded as shown in a and b. The divalent metal ions identified in the tertiary structure are shown as green balls. c A ribbon view of the 2 Å structure of the four-way junctional twister-sister ribozyme color-coded according to helical segments observed in the tertiary structure. All highly conserved residues are labeled by red circles. Long-distance interactions observed in the tertiary structure are labeled with dashed lines named T1 (in red) and T2 (in black). The cleavage site step was shown by a red arrow.
conserved stem P1 \(^{11}\). A single-molecule FRET study on twister folding rationalizes this behavior by revealing that the active site-embracing pseudoknot fold is preserved in the shortened ribozyme and also in the cleaved 3'-product RNA that both lack P1 \(^{12}\).

More recently, the structural studies have been extended to the pistol ribozyme \(^{3, 18}\), where there is good consensus on both the overall folding topology and alignment of catalytic residues at the cleavage site. This may reflect the anchoring of both pyrimidine and purine at the pyrimidine–purine cleavage step in the pre-catalytic conformation of the pistol ribozyme.

Twister and twister-sister appear to be related ribozymes based on similar helical connectivities and common conserved residues positioned in similarly aligned hairpin and internal loops \(^5\). We report here the structure of a four-way junctional twister-sister ribozyme and discuss possible/putative roles of selected nucleobases and hydrated Mg\(^{2+}\) ions observed at the active site for cleavage chemistry. We also compare our structure of twister-sister ribozyme composed of a four-way junctional fold (this study), with a recently reported structure of the twister-sister ribozyme composed of a three-way junctional fold \(^{17}\) and note unanticipated differences in one long-range interaction and in alignments in the catalytic pocket between the two structures. Finally, a comparison between previously reported structures of the twister ribozyme \(^{9, 11}\) and the structure of the twister-sister ribozyme (this study) highlights similarities in structural alignments in the catalytic pocket that may indicate shared concepts of these two related ribozymes to mediate cleavage by involving a guanine–scissile phosphate interaction, a splayed-apart conformation of the cleavage site, and involvement of—although to different extent—hydrated divalent Mg\(^{2+}\) ions.

**Results**

**Twister-sister ribozyme construct and cleavage propensity.** We screened a large number of chemically synthesized two-stranded and three-stranded constructs of the twister-sister ribozyme, before identifying a two-stranded construct composed of a four-way junctional fold shown schematically in Fig. 1a, that yielded diffraction quality crystals. Cleavage assays on the wild-type two-stranded twister-ribozyme construct established site-specific cleavage at the C62–A63 step under 2 mM divalent cation conditions with fastest cleavage observed for Mn\(^{2+}\), followed by Mg\(^{2+}\) and Ca\(^{2+}\); cleavage was slowest for Sr\(^{2+}\) (Supplementary Fig. 1b).

**Tertiary fold of the twister-sister ribozyme.** For crystallographic studies, we introduced dC62 into the sequence of the longer substrate strand of the two-stranded construct, so as to prevent cleavage at the known C62–A63 cleavage site of the twister-sister ribozyme \(^3\). The structure was solved at 2 Å resolution using phases determined for crystals soaked with Ir(NH\(_3\))\(^3+\) (X-ray statistics are listed in Supplementary Table 1). There are two molecules of the twister-sister ribozyme in each asymmetric unit (space group: P2\(_2\), 2\(_2\), 2), with both exhibiting well-defined electron density, while also superpositioning well with an rmsd = 0.27 Å. A schematic of the tertiary fold of the twister-sister ribozyme is shown in Fig. 1b, whereas the three-dimensional structure in a ribbon representation is shown in Fig. 1c.

As shown in the schematic of the secondary fold in Fig. 1a, our two-stranded construct of the twister-sister ribozyme is composed of stems P1 (in orange), P2 (in blue) and P3 (in cyan), and stem-loops P4-SL4 (in magenta) and P5-SL5 (in green). Loop L1 is positioned between stems P1 and P2, whereas a four-stem junction is formed at the intersection of stems P2, P3, P4, and P5. Highly conserved residues are shown in red circles and are distributed between L1 and SL4 loop segments, which are spatially separated in the secondary structure (Fig. 1a) but brought into close proximity in the tertiary structure (Fig. 1d; residues labeled in red) of the twister-sister ribozyme.

As shown in the schematic of the tertiary fold of our construct of the twister-sister ribozyme (Fig. 1b), stems P1, P2, and P3 form one continuous helix (Supplementary Fig. 2a), whereas stems P4 and P5 form a second continuous helix (Supplementary Fig. 2b), with elements of these two continuous helices meeting at the junctional site (Fig. 1b, d). Notably, SL4 projecting from continuous helix P5–P4 is positioned in the minor groove of the partially zippered-up L1 loop segment associated with the continuous helix P1–P2–P3 helix (Fig. 1b, c). The stacking between terminal base pairs of extended stems P1 and P2 are shown in Supplementary Fig. 2c and d, those between extended stems P2 and P3 are shown in Supplementary Fig. 2e and f, whereas those between terminal base pairs of stems P4 and P5 are shown in Supplementary Fig. 2g and h.

We observe additional pairing on formation of the tertiary fold of the twister-sister ribozyme (Fig. 1b). Thus, loop L1 partially zippers up, whereby stem P1 is extended through trans non-canonical G5–U64 (Supplementary Fig. 3a) and trans sugar edge-Hoogsteen C6–A63 (Supplementary Fig. 3b) pairing involving G5 and G6, whereas stem P2 is extended by the Watson–Crick A8–U61, which forms part of a major groove aligned G59–(A8–U61) base triple (Supplementary Fig. 3c) and the Watson–Crick G9–C60 pairing, accompanied by extrusion of G59. Note that all residues of the base triple, namely A8, G59, and U61 are highly conserved in the twister-sister ribozyme. SL4 also partially zippers up, whereby stem P4 is extended through the Watson–Crick G29–C37 pairing involving highly conserved residues and the trans Watson–Crick–Hoogsteen U30–A34 pairing (Supplementary Fig. 3d), accompanied by extrusion of G35 and U36. In addition, stem P3 is extended by the cis Watson–Crick–Watson–Crick A14–C23 (Supplementary Fig. 3e) and Watson–Crick G13–C24 pairing at the four-way junctional site of the twister-sister ribozyme.

We have identified a large number of hydrated Mg\(^{2+}\) cations distributed throughout the tertiary fold of the twister-sister ribozyme (green balls, Fig. 1c), with divalent cation Mg\(^{2+}\) occupancy confirmed followed substitution by Mn\(^{2+}\) and monitoring the latter’s anomalous diffraction characteristics (Supplementary Fig. 4a). Our studies have focused on seven Mg\(^{2+}\) cation sites, all of which have been validated by Mn\(^{2+}\) replacement, as shown for divalent cations labeled M1 to M4 in Supplementary Fig. 4b and c, for M5 in Supplementary Fig. 4d and e, and for M6 to M7 in Supplementary Fig. 4f and g.

**Structural alignments at the four-way junctional site.** At the junctional site of the twister-sister ribozyme, both splayed-apart C24–G25 (junctional J3 segment) and G55–G56 (junctional J52 segment) steps (Fig. 1b) are brought into close proximity, with a hydrated Mg\(^{2+}\) (labeled M5) bridging opposingly aligned J34 and J52 junctional segments (Fig. 2a). A network of hydrogen bonds involving C24–G25, G55–G56, looped out highly conserved G35 and hydrated M5 stabilizes this junction site (Fig. 2a, b). M5 forms symmetrical inner-sphere coordination to the non-bridging pro-S\(_p\) oxygen of the C24–G25 phosphate and the pro-S\(_s\) oxygen of the G55–G56 phosphate, and to four water molecules (Fig. 2b). Notably, of the five nucleotides (C24, G25, G55, G56, and G35) involved in the alignment at this junctional site, only C24 adopts a C2′-endo sugar pucker conformation. C24 and G56 form terminal stacking interactions between stems P2 and P3 (Fig. 2a; Supplementary Fig. 2e, f), whereas G25 and G55 form terminal stacking interactions between stems P4 and P5 (Fig. 2a; Supplementary Fig. 2g, h).

The A33–A34 step of stem-loop SL4 (composed of loop G31–C32–A33 segment closed by a non-canonical U30–A34
pair) forms a long-range interaction with extruded A7 from the zippered-up loop L1 (see red dashed line labeled T1 in Fig. 1a), whereby A7, which adopts a C2′-endo sugar pucker conformation, is sandwiched between adjacent A33 and A34 (Fig. 2c), and anchored in place by forming a non-canonical A7•G31 pair (Fig. 2d). Notably, the G31–C32–A33 segment and A7 adopts a stable GNRA hairpin loop fold. The major groove base edge of highly conserved residue A33 forms a hydrogen bond with the 2′-OH of G31, whereas its minor groove base edge forms a hydrogen bond with the 2′-OH of A7, contributing to the hydrogen bond network stabilizing the interaction between A7, G31, and A33 (Fig. 2d).

Further, looped out bases G35 and U36 emanating from zippered-up SL4 loop adopt a stacked alignment and are positioned in the major groove of stacked bases of stem P2 and zippered-up loop L1, thereby forming a second long-range alignment (Fig. 2e; see black dashed line labeled T2 in Fig. 1a), with highly conserved G35 anchored in place through formation of a major groove aligned G35•(G56–C12) base triple (Fig. 2f). In addition, we found that the carbonyl groups of U36 forms two hydrogen bonds with the inner-sphere waters of two hydrated Mg2+ ions, in which one is coordinated with O6 of G59 and the other is coordinated with the non-bridging phosphate oxygens of residues A8 and A9 (g).
Structural alignments at the C62–A63 cleavage step. Bases C62 and A63 at the cleavage site are splayed apart, with C62 directed outwards and A63 directed inwards into the ribozyme fold (Fig. 3a). C62 appears to adopt a mixture of syn and anti conformations reflective of flexibility about its glycosidic bond, whereas A63 is anchored in place in an anti conformation by stacking between bases (Fig. 3b), with its N7 atom hydrogen bonded to the 2'-OH of C6 (Fig. 3c). We have modeled the oxygen corresponding to 2'-OH at position C62 and measure a distance of 3.8 Å between the 2'-O and the phosphorus of the C62–A63 cleavage step and an angle of 71° for 2'-O of C62 relative to the P-O5' bond (off-line alignment) at the cleavage step.

The non-bridging oxygens of the C62–A63 scissile phosphate are anchored in place through hydrogen bonding to the N1H of G5 (coordinate to pro-Sp oxygen with heteroatom separation of 2.7 Å) and to an inner-sphere water of a hydrated Mg2+ labeled M1 (H-bonded to pro-Rp oxygen with heteroatom separation of 2.9 Å; M1 is also coordinated to pro-Rp oxygen of U61 (2.1 Å) and pro-Rp of C60 (2.2 Å); it is further coordinated to pro-Sp of G50' from a symmetrical molecule (2.3 Å)) (Fig. 3c). The modeled 2'-OH of C62 (red arrow, Fig. 3c, d) is positioned for hydrogen bonding to an inner-sphere water of hydrated Mg2+-labeled M2 (heteroatom separation of 3 Å) (black arrow, Fig. 3c, d).

The octahedral coordination geometries and hydrogen bond networks of a set of three hydrated Mg2+ cations labeled M3, M1, and M4 within a single twister-sister ribozyme are highlighted in Fig. 3e. Both M1 and M4 are also coordinated to groups in a symmetry-related molecule in the crystal lattice (not shown).

Cleavage assays on twister-sister ribozyme mutants. We have undertaken cleavage assays in the context of a two-stranded construct of the twister-sister ribozyme (Fig. 4a; Supplementary Fig. 1a). Cleavage data on the wild-type twister-sister ribozyme under 2 mM Mg2+, Mn2+, Ca2+, and Sr2+ divalent cation conditions are compared in Supplementary Fig. 1b, with cleavage enhanced under Mn2+ conditions relative to the other divalent conditions.
and C24dC/G55dG) (Fig. 5c, f, j) in activity. By contrast, we observe enhanced cleavage for C62A and C62G (Fig. 5d, e), with the focus in this paper on metal sites labeled M1 to M7, positioned either at the junctional or cleavage sites. We have undertaken cleavage assays on base, sugar, and backbone modified twister-sister ribozymes that have been undertaken on a three-stranded construct shown in Fig. 5a and Supplementary Fig. 1c, with the shorter individual strands facilitating site-specific incorporation of modifications. Cleavage assays on the three-stranded construct under 2 mM divalent cation conditions indicate no cleavage under Mg2+ conditions, with the data on G5A, G5U, G5C, and G5I shown in Fig. 4c–f. Although cleavage is essentially retained for the G5I substitution (Fig. 4f), there is essentially complete loss of cleavage for G5U and G5C substitutions (Fig. 4d, e) and very minor cleavage for G5A (Fig. 4c). We also performed the cleavage assays of the substitutions C62U (Fig. 4g), C62A (Fig. 4h), and C62G (Fig. 4i), with the first two mutants displaying pronounced cleavage activity, whereas the third mutant shows somewhat reduced cleavage activity.

Further comprehensive cleavage assays on base, sugar, and backbone modified twister-sister ribozymes have been undertaken on a three-stranded construct shown in Fig. 5a and Supplementary Fig. 1c, with the shorter individual strands facilitating site-specific incorporation of modifications. Cleavage assays on the three-stranded construct under 2 mM divalent cation conditions indicate no cleavage under Mg2+ conditions, with the data on G5A, G5U, G5C, and G5I shown in Fig. 4c–f. Although cleavage is essentially retained for the G5I substitution (Fig. 4f), there is essentially complete loss of cleavage for G5U and G5C substitutions (Fig. 4d, e) and very minor cleavage for G5A (Fig. 4c). We also performed the cleavage assays of the substitutions C62U (Fig. 4g), C62A (Fig. 4h), and C62G (Fig. 4i), with the first two mutants displaying pronounced cleavage activity, whereas the third mutant shows somewhat reduced cleavage activity.

Given these observations, we have undertaken cleavage assays on mutations of the three-stranded construct of the twister-ribozyme under 10 mM Mg2+ conditions, with the data on G5A, G5U, G5C, and G5I shown in Fig. 4c–f. Although cleavage is essentially retained for the G5I substitution (Fig. 4f), there is essentially complete loss of cleavage for G5U and G5C substitutions (Fig. 4d, e) and very minor cleavage for G5A (Fig. 4c). We also performed the cleavage assays of the substitutions C62U (Fig. 4g), C62A (Fig. 4h), and C62G (Fig. 4i), with the first two mutants displaying pronounced cleavage activity, whereas the third mutant shows somewhat reduced cleavage activity.

Further comprehensive cleavage assays on base, sugar, and backbone modified twister-sister ribozymes have been undertaken on a three-stranded construct shown in Fig. 5a and Supplementary Fig. 1c, with the shorter individual strands facilitating site-specific incorporation of modifications. Cleavage assays on the three-stranded construct under 2 mM divalent cation conditions indicate no cleavage under Mg2+ conditions, with the data on G5A, G5U, G5C, and G5I shown in Fig. 4c–f. Although cleavage is essentially retained for the G5I substitution (Fig. 4f), there is essentially complete loss of cleavage for G5U and G5C substitutions (Fig. 4d, e) and very minor cleavage for G5A (Fig. 4c). We also performed the cleavage assays of the substitutions C62U (Fig. 4g), C62A (Fig. 4h), and C62G (Fig. 4i), with the first two mutants displaying pronounced cleavage activity, whereas the third mutant shows somewhat reduced cleavage activity.

Discussion

We first discuss our four-way junctional structure of the twister-sister ribozyme in the context of mutational studies and then compare and note unanticipated differences in the active site architecture with a recently solved three-way junctional structure of the twister-sister ribozyme. We next compare our structures of the twister9, 11 and twister-sister (this study) ribozymes to consider the merits of their designations as related ribozymes.

We have observed a large number of binding sites for hydrated metal cations in the structure of the twister-sister ribozyme (Fig. 1c), with the focus in this paper on metal sites labeled M1 to M7, positioned either at the junctional or cleavage sites. We have undertaken Mn2+ soak experiments to use the anomalous properties of these ions to definitively differentiate between monovalent and divalent cation sites. These experiments (examples shown for M1 to M7 in Supplementary Fig. 4b–g) unambiguously establish that M1 to M7 are all divalent cations, with the observed octahedral geometry for M1 to M7 with metal to water oxygen distances of 2 Å, consistent with hydrated Mg2+ for these metal sites (M1–M4 are shown in Fig. 3c–e; M5 is shown in Fig. 2a and b, and M6–M7 are shown in Fig. 2e and g).

One notable feature of the junctional alignment is the network of hydrogen-bonding interactions involving hydrated Mg2+ cation M5-mediated stitching together of elements from stems P2, P3, P5, and stem-loop P4 SL4 (Fig. 2a, b). Notably, the 2′OH groups of C24 and G55, form symmetrical hydrogen bonds to the opposite non-bridging phosphate oxygens, resulting in involvement of all four non-bridging phosphate oxygens of the two M5-bridged phosphates in interstrand stabilizing interactions (Fig. 2a, b). Consistent with such an alignment, cleavage activity was abolished for the C24dC/G55dG-substituted twister-sister ribozyme.
Fig. 5 Self-cleavage of the three-stranded twister-sister ribozyme. a The secondary structure of the three-stranded twister-sister ribozyme used in the cleavage assays. Rc’ denotes the 17-nt substrate (green); C1 (blue) and C2 (yellow) denote 6-nt and 11-nt cleavage products. b–k HPLC traces following cleavage activity of wild-type ribozyme (b) and mutants G5A (c), C62A (d), C62G (e), A63U (f), A7U (g) and A8U (h), C6dC (i) and C24dC, G55dG (j), and C60dCmP (k). Cleavage activity analyzed at 55 µM RNA each strand; 10 mM MgCl₂, 100 mM KCl, 30 mM HEPES, pH 7.5, 23 °C. HPLC conditions: Dionex DNA PAC column (4 × 250 mm²), 80 °C, 1 mL min⁻¹, 0–60% buffer B in 45 min. Buffer A: Tris–HCl (25 mM), urea (6 M), pH 8. Buffer B: Tris–HCl (25 mM), urea (6 M), NaClO₄ (0.5 M), pH 8
Mg$^{2+}$ cations M6 and M7 (as shown in Fig. 2g), which were highly conserved residues were labeled with red circles. The long-distance interactions are shown with dash line labeled as T1 (in red) and T2 (in black). The cleavage site is shown with a red arrow. b Expanded view of the cleavage site. C54 is stacked with A55, and this stacked pair is sandwiched between bases A9 and U57. c An inner-sphere water of a hydrated Mg$^{2+}$ is within hydrogen-bonding distance of the modeled 2'-OH C54. The enol tautomer form of U56 may form a hydrogen bond with the pro-S$_p$ non-bridging phosphate oxygen of the cleavage step.

(Fig. 5). Similarly, a trio of sequentially aligned hydrated Mg$^{2+}$ cations (M3, M1, and M4) involved primarily in non-bridging phosphate oxygen coordination form a stabilizing network bridging opposingly positioned G5–C6 and C58–G59–C60–U61 segments proximal to the C62–A63 cleavage site (Fig. 3e).

Nucleotides A7, G35, U36, and G59 are looped out of the stacked helical-rich architecture of the twister-sister ribozyme. Nevertheless, they are all involved in long-range pairing and stacking interactions stabilizing and interdigitating together the fold of the twister-sister ribozyme. Thus, extruded A7 intercalates between A33 and A34 (Fig. 2c), and in addition, forms base–sugar and base–base hydrogen bonds to A33 and G31 (Fig. 2d). Similarly, extruded G35 and G59 form major groove aligned G35•(C12–G56) (Fig. 2f) and G59•(A8–U61) base triples (Supplementary Fig. 3c). In addition, U36 forms extensive interactions with G59 and the A8–G9 step through two hydrated Mg$^{2+}$ cations M6 and M7 (as shown in Fig. 2g), which were confirmed by the anomalous signal collected with Mn$^{2+}$-soaked crystals (Supplementary Fig. 4f, g). Interestingly, one metal designated as M8, that is coordinated to N7 of G35 has strong anomalous signal in the Mn$^{2+}$-soaked crystals (Supplementary Fig. 4f, g), whereas its counterpart is not observed in the native crystal, which may explain why the three-stranded native twister-ribozyme construct has better cleavage activity with Mn$^{2+}$ than Mg$^{2+}$ under 2 mM divalent cation conditions (Supplementary Fig. 1c, d).

The non-conserved A7 residue can be substituted by U without much loss of activity (Fig. 5g). A greater impact on activity is observed for the replacement of the highly conserved A8 by U, which reduces the extent of cleavage but not completely abolishes it (Fig. 5h). This finding is consistent with the possibility of forming a U8•U61 non-canonical base pair, without disturbing the interaction between G59 and U61 of the base triple (Supplementary Fig. 3c).

We have also monitored the importance of mutating C62 and A63 at the C62–A63 cleavage step in the twister-sister ribozyme. Replacement of flexible C62 by U, A, or G retains cleavage activity (Figs. 4g–i and 5d, e), whereas replacement of anchored A63 by U results in complete loss in activity (Fig. 5f). It appears that the A63U mutation most likely disrupts both the stacking and hydrogen-bonding interactions that anchor A63 within the fold of the twister-sister ribozyme. Furthermore, we probed the trans sugar edge-Hoogsteen C6•A63 pair (Supplementary Fig. 3b) by deletion of the 2'-OH of G6; indeed, the C6dC mutant exhibited a reduced extent of cleavage (Fig. 5i).

We made G5 mutations, given that N1H of G5 is directed to the pro-S$_p$ non-bridging phosphate oxygen at the cleavage step (Fig. 3c, d) and all its ring heteroatoms are involved in hydrogen-bonding interactions (Fig. 3e). Notably, the G5A mutation results in very minor cleavage (Figs. 4c and 5c), suggesting that G5, through its NIH amide functionality (would be N1 imino in adenine), might have an important role for the cleavage chemistry although its direct participation in catalysis remains to be proven, given that it is not absolutely conserved. Similarly, replacement of purine G5 by pyrimidines U or C results in nearly complete loss of activity (Fig. 4d, e). By contrast, the G5I mutant shows cleavage activity (Fig. 4f) comparable to wild-type, implying that disruption of the hydrogen-bonding potential of the 2-amino group of G5 has no effect on cleavage activity.

Earlier studies established that the cleavage rate of the twister-sister ribozyme increased on raising the pH from 5 to 7 before plateauing at neutral pH and also exhibited a steep dependence on Mg$^{2+}$ concentration before plateauing out at 1 mM Mg$^{2+}$ concentration. In the absence of Mg$^{2+}$, no cleavage was detectable in the presence of Co(NH$_3$)$_6$$^{3+}$, nor was cleavage observed...
for monovalent cations (except Li$^+$ at very high concentrations). Hydrated Mg$^{2+}$ cation M1, with an inner-sphere-coordinating water donating a hydrogen bond to the pro-$R_P$ non-bridging oxygen of the scissile phosphate (Fig. 3c, e), is anchored in place through direct coordination to three non-bridging phosphate oxygens. These are the pro-$R_P$ oxygen of U61 and the pro-$S_P$ oxygen of G50' from a symmetrical molecule. To learn more about the potential relevance of the M1 ion from a mechanistic perspective, we synthesized RNA with a methylphosphonate backbone unit between C60 and U61 (C60dCmP) that is expected to interfere with the transition-state conformation. We did not observe an inline alignment (2$'$-OH of C62, as suggested previously), the interaction labeled T2, where it forms a Watson–Crick base triple with the major groove edge of the G56–C12 pair (Fig. 2f) and U36 forms extensive hydrogen-bonding interactions. By contrast, in the published three-way junctional structure of the twister-sister ribozyme, only one base-labeled G27 is involved in the interaction labeled T2, where it forms a Watson–Crick base pair with the major groove edge of the G56–C12 pair (Fig. 2f), and U36 forms extensive hydrogen-bonding interactions with highly conserved base G59, and the non-bridging phosphate oxygens of A8 and G9 through two hydrated Mg$^{2+}$ ions (Fig. 2g). The alignments within the catalytic pocket of the twister-sister ribozyme for cleavage at the C–A step in our four-way junctional structure of the twister-sister ribozyme shown in Fig. 3b, can be compared with its counterpart in the published three-way junctional structure of the twister-sister ribozyme shown in Fig. 6b and c. We note some similarities between these two structures of...
the twister-sister ribozyme. For instance, the modeled 2′-OH of C
at the cleavage site is within hydrogen-bonding distance of an
inner-sphere water of a hydrated Mg2+ cation in both structures
(Figs. 3c, d and 6c).

By contrast, we note significant differences in orientation as
reflected in the splayed-apart alignment of the C–A step, with C
adopting flexible and A adopting anchored alignments in our
structure (Fig. 3b–d), in contrast to the unanticipated stacked
alignment of the C–A step, with C adopting anchored and A
adopting flexible alignments in the published structure27
(Fig. 6b, c). The pro-Sp non-bridging phosphate oxygen at the
C–A step is hydrogen bonded to N1H of G5 in our four-way
junctional (4-wj) structure (Fig. 3c–e), whereas hydrogen bonding
to U56, which is directed towards the pro-Sp non-bridging
phosphate oxygen, could only form for the enol tautomomer of U56
in the three-way junctional (3-wj) structure17 (Fig. 6c). In addi-
tion, the pro-Rp non-bridging oxygen of the C–A scissile phos-
phate forms a hydrogen bond with an inner-sphere water of
hydrated Mg2+ cation M1 in 4-wj structure (Fig. 3c, e), whereas
no such hydrated Mg2+ site was observed in the 3-wj structure17
(Fig. 6c). Finally, off-line cleavage alignments are observed both
in the 4-wj structure (O2′ P-O5′ angle of 71°) and in the 3-wj
structure (67°)17 for the pre-catalytic state of the twister-sister
ribozyme.

These significant differences in the alignments within the cat-
alytic pocket between the four-way (our structure) and three-
way15 junctional folds of the twister-sister ribozyme were unex-
pected and imply that secondary structure and/or crystallographic
packing can influence the topology of the catalytic pocket in these
pre-catalytic conformations, without impacting significantly on
the global tertiary fold of the ribozyme. In particular, we point out
that the one nucleotide larger L1 segment of the three-way
junctional (Fig. 6a) compared to the four-way junctional (Fig. 1a)
twister-sister ribozyme may contribute to the structural differ-
ences at the cleavage site.

In the following, we compare our structures of the twister
(Fig. 7)9, 11 and twister-sister (Fig. 3) (this study) ribozymes and
point out alignment similarities within the catalytic pocket of
both ribozymes. The catalytic pocket alignments at the U–A
cleavage step (U 2′-OH replaced by U 2′-OCH3) for our 2.6 Å
structure of the twister ribozyme11 shown in Fig. 7c is com-
pared with that at the C-A cleavage step (C 2′-OH replaced by C2′H)
in our 2 Å structure of the twister-sister ribozyme (this study)
shown in Fig. 3c and d. Long-range loop–loop interactions
involving conserved residues define the tertiary folds and catalytic
pocket alignments in twister-sister (Supplementary Fig. 6a, b) and
twister (Supplementary Fig. 6c, d) structures. Notably, the
pyrimidine–purine step is splayed apart, with the pyrimidine
directed outwards and adopting a flexible alignment, whereas
the purine is directed inwards and anchored in position through
base-stacking interactions in both ribozyme structures. The N1H
of a guanine is hydrogen bonded to one of the non-bridging
phosphate oxygens (pro-Rp, in twister (Fig. 7c) and pro-Sp in
twister-sister ribozymes (Fig. 3c, d)), whereas a hydrated Mg2+
is coordinated to the other non-bridging phosphate oxygen
(direct coordination to pro-Sp in twister (Fig. 7c) and inner-
sphere water-mediated coordination to pro-Rp in twister-sister
(Fig. 3c, d)).

The outwardly pointing pyrimidine base of the
pyrimidine–purine step at the cleavage site adopts a flexible
alignment in our structures of the twister and twister-sister
ribozymes and can be replaced by adenine and guanine (as well as
cytosine and uracil, respectively) in both ribozymes, thereby
retaining high cleavage activity8. This implies that the positioning
of the pyrimidine nucleoside in the pre-catalytic state structures
may not reflect its alignment in the transition state, in contrast to
the positioning of the purine nucleoside, whose alignment is
stabilized by stacking interactions, and both non-bridging oxy-
gens of the scissile phosphate, which are anchored in place
through hydrogen bonding, in the pre-catalytic structures of both
ribozymes. Indeed, replacement of this adenine by another base
in both ribozymes results in loss of cleavage activity7. It is not
clear at this time as to how much weight should be placed on
the observation of a hydrated Mg2+ (M2) whose inner shell water
is positioned to hydrogen bond with the modeled 2′-OH of C62
(given the flexibility of the pyrimidine base) in our structure of
the twister-sister ribozyme (Fig. 3c, d), given that a hydrated
Mg2+ cation was not observed in the same position in our
structure of the twister ribozyme (Fig. 7c).

It is also not yet clear as to how much weight should be placed
on the Mg2+ (M1) whose inner shell water is positioned to
hydrogen bond with the scissile phosphate oxygen (Fig. 3c, d)
that we see for our structure of the twister-sister ribozyme. For
twister, we found direct coordination of Mg2+ to the scissile
phosphate (Fig. 7c); this interaction was probed with enanti-
omerically pure thiophosphate substrates and resulted in an only
about twofoldrio rescues (with Mn2+ or Cd2+11), suggesting that
divalent metal ions are likely to be minor contributors to the
chemical mechanism despite their possible direct interaction in
solution18, 19. For twister-sister where we only observe the scissile
phosphate interacting via a H2O that is inner-sphere coordinated
to Mg2+ (Fig. 3c, d), rescue experiments with scissile thiopho-
phosphates might encounter limitations. In our view, the above
listed similarities within catalytic pocket alignments centered about
the to-be-cleaved phosphate group in our structures are supportive of
the designations of twister3 and twister-sister (this study) as
related ribozymes4.

Notably, of previously characterized small self-cleaving ribo-
zymes, the HDV (hepatitis delta virus) ribozyme employs a
combination of nucleobase and metal-ion catalysis20–22, the GlmS
ribozyme uses a cofactor (glucosamine-6-phosphate) for cata-
lysis23–25 and others including hairpin26, 27, hammerhead28–30,
and VS ribozymes31 mainly utilize nucleobases as catalytic
groups32. With the now available structures of the twister-sister
ribozyme, it appears likely that this ribozyme reveals additional
facets of RNA catalysis for phosphodiestereavage. How
twister-sister employs and combines the main catalytic strategies
(α, β, γ, δ catalysis)33, however, is not clear and warrants further inves-
tigations. X-ray crystallography has played a key role in defining
the overall folding topology of small self-cleaving ribozymes,
thereby allowing selective mutation studies on catalytic pocket
residues to evaluate their impact on cleavage chemistry. Never-
theless, it should be noted that to prevent cleavage chemistry
during crystallization of the ribozymes, the 2′-OH of the
nucleotide preceding the cleavage step is generally replaced by 2′-
H or 2′-OCH3, with the potential for such modifications to
perturb alignments at the cleavage site.

Further progress towards an improved understanding of me-
chanistic aspects of cleavage chemistry will depend on moving
forward from available structures of pre-catalytic states of the
newly identified self-cleaving ribozymes that generally exhibit
significant departures from inline alignments at the cleavage site,
to structures of transition-state-like vanadate complexes, similar
to those reported previously for the hairpin34 and hammerhead35
ribozymes, to get a more complete and relevant overview of the
potential diversity of catalytic mechanisms adopted by small self-
cleaving ribozymes.

Methods
Solid-phase synthesis of oligonucleotides. All oligonucleotides were synthesized
on a ABI 392 Nucleic Acid Synthesizer using 2′-O-TOM standard RNA nucleoside
phosphoramidite building blocks (ChemGenes) and polystyrene support (GE
phosphoramidite building blocks (ChemGenes) and polystyrene support (GE
www.nature.com/naturecommunications
DOI: 10.1038/s41467-017-01276-y
Healthcare, Prime Support 5G, 300 µmol per g; PS 200). 5′-O-(4,4′-dimethoxytrityl)-2′-deoxycytidine phosphoramidite was purchased from ChemGenes. Reaction conditions: detritylation (90s) with dichloroacetic acid/2-dichloroethane (4/96); coupling (2.0 min) with phosphoramidites/acetidinium (0.1 M x 130 µL) and benzylthiotetrazole/acetidinium (0.3 M x 360 µL); capping (3 x 0.4 min, Cap A/Cap B = 1/1) with Cap A: 4-(dimethylamino)pyridine in acetidinium (0.5 M) and Cap B: Ac2O/sym-collidine/acetonitrile (2/3/5); oxidation (1.0 min) with I2 (20 mM) in THF/pyridine/H2O (35/10/5). The solutions of amidites and tetrazole, and acetidinium were dried over activated molecular sieves (3 Å) overnight.

Deprotection of oligonucleotides. The solid support was reacted with MeNH2 in EtOH (33%, 0.5 mL) and MeNH2 in water (40%, 0.5 mL) for 7 h at room temperature. The supernatant was removed from and the solid support was washed with THF/water (1/1, v/v). The combined supernatant and the washings were evaporated to dryness and the resulting residue was treated with tetrabutylammonium fluoride trihydrate (TBAF·3H2O) in THF (1 M, 1 mL) at 37 °C overnight to remove the 2′-O-silyl protecting groups. The reaction was stopped by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 1 mL). The volume of the solution was reduced and the solution was desalted using a size exclusion column (GE Healthcare, HiPrep 26/10 Desalting; 2.6 × 10 cm; Sephadex G25) eluting with H2O. The collected fraction was evaporated to dryness and dissolved in 1 ml H2O. (GE Healthcare, Primer Support 5G, 300 µmol per g; PS 200). The crude RNA after deprotection was analyzed by anion-exchange chromatography on a Dionex DNA PAC PA-100 column (4 mm x 250 mm) at 80 °C. Flow rate: 1 ml/min. 25 mM Tris-HCl (pH 8.0), 6 M urea; eluant: B: 25 mM Tris-HCl (pH 8.0), 0.5 M NaClO4, 6 M urea; gradient: 0–80% B in A within 45 min, UV detection at 260 nm.

Puriﬁcation of RNA. The crude deprotected RNA was purified on a semi-preparative Dionex DNA PAC PA-100 column (9 mm x 250 mm) at 80 °C with flow rate 2 ml/min. RNA containing fractions were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1 M NaOAc, pH 5.2, 0.15 M CaCl2, and 30% MPD and supplemented with 20 mM Ir(NH3)6Cl3+Cl− or 50 mM MnCl2·H2O, quickly transferred into a cryoprotectant solution containing 0.1 M NaOAc, pH 5.2, 0.15 M (Et3NH)+HCO3−, 22% iso-propanol or MPD. The typical crystal dimensions of the native twister-ribozyme were 0.22 × 0.22 × 0.21 mm; 0.15 × 0.15 × 0.15 mm for the two-strand cleavage structure, followed by refinement of the structure with PHENIX37 with a final Rwork/Rfree = 0.22/0.24 (Table 1). The coordinate error estimate with maximum-likelihood method in PHENIX37 is 0.36 Å. The 2Fobs−Fcalc electron density map (contoured at 1σ) was overlaid with the native twister-ribozyme structure as a starting model. We observe metal-O coordination distances of 2 Å characteristic of Mg2+ coordination, rather than the longer 2.4 Å distance characteristic of Ca2+ coordination, given the context that Ca2+ was the predominant divalent cation present in our crystallization buffers. The X-ray data statistics of the native, Ir (NH3)6Cl3+-containing and Mn2+ soaked crystals are listed in Table 1. The structure in figures were prepared using program PyMOL (http://www.pymol.org/).

Cleavage assays. Aliquots from aqueous millimolar stock solutions of the two or three RNA strands (Ra, Rb or Ra’, Rb, Rc’) were mixed and lyophilized. After addition of reaction buffer (30 mM HEPES, pH 7.5, 100 mM KCl, MgCl2 concentrations as indicated) to yield a final concentration of c(RNA) = 35 mM (each strand) in a total volume of 20 µL, the reaction was stopped by the addition of EDTA solution (20 µL; 3 mM) after 1, 10, 45, and 120 min, stored at 4°C, and subsequently analyzed by anion-exchange HPLC (analytical Dionex DNA Pac column) using the conditions as described above. For the two-strand cleavage assay, the reaction mixture was briefly heated to 70°C (~20 s) immediately after dissolving of the RNA to support proper annealing.

Data availability. The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the following accession codes: 5T85 for dC-d22-containing four-ribozyme, 2M7P for dC-d22-containing four-ribozyme, and 3X87 for same ribozyme crystals soaked in Mn2+ solution. Other data are available from the corresponding authors upon reasonable request.
References

1. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, L. R. & Bruneng, G. Autolitic processing of dimeric plant virus satellite RNA. Science 231, 1577–1580 (1986).
2. Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. Nucleic Acids Res. 14, 3627–3640 (1986).
3. Roth, A. et al. A widespread self-cleaving ribozyme class is revealed by bioinformatics. Nat. Chem. Biol. 10, 56–60 (2014).
4. Weinberg, Z. et al. New classes of self-cleaving ribozymes revealed by comparative genomics analysis. Nat. Chem. Biol. 11, 606–610 (2015).
5. Jimenez, R. M., Polanco, J. A. & Luptak, A. Chemistry and biology of self-cleaving ribozymes. Trends Biochem. Sci. 40, 648–661 (2015).
6. Breaker, R. R. Mechanistic debris generated by twister ribozymes. ACS Chem. Biol. 12, 886–891 (2017).
7. Gebetsberger, J. & Micura, R. Unwinding the twister ribozyme: from structure to mechanism. Wiley Interdiscip. Rev. RNA 8, e1402 (2017).
8. Liu, Y., Wilson, T. J., McPhee, S. A. & Lilley, D. M. Crystal structure and mechanistic investigation of the twister ribozyme. Nat. Chem. Biol. 10, 739–744 (2014).
9. Ren, A. et al. In-line alignment and Mg2+ coordination at the cleavage site of the env22 twister ribozyme. Nat. Commun. 5, 5534 (2014).
10. Eiler, D., Wang, J. & Stietz, T. A. Structural basis for the fast self-cleavage reaction catalyzed by the twister ribozyme. Proc. Natl Acad. Sci. USA 111, 13028–13033 (2014).
11. Kosutic, M. et al. A mini-twister variant and impact of residues/cations on the phosphodiester cleavage of this ribozyme class. Angew. Chem. Int. Ed. Engl. 54, 15128–15133 (2015).
12. Vusurovic, N., Altman, R. B., Terry, D. S., Micura, R. & Blanchard, S. C. Pseudoknot formation seeds the twister ribozyme cleavage reaction coordinate. J. Am. Chem. Soc. 139, 8186–8193 (2017).
13. Gaines, C. S. & York, D. M. Ribozyme catalysis with a twist: active state of the RNA pseudoknot network in the active site. J. Am. Chem. Soc. 138, 3058–3065 (2016).
14. Ucsiik, M. N., Bevilacqua, P. C. & Hammes-Schiffer, S. Molecular dynamics study of twister ribozyme: role of Mg2+ ions and the hydrogen-bonding network in the active site. Biochemistry 55, 3834–3846 (2016).
15. Ren, A. et al. Pseudoknot ribozyme adopts a pseudoknot fold facilitating site-specific in-line cleavage. Nat. Chem. Biol. 12, 702–708 (2016).
16. Nguyen, L. A., Wang, J. & Stietz, T. A. Crystal structure of Pseudoknot formation seed the Pseudoknot ribozyme cleavage reaction. Proc. Natl Acad. Sci. USA 114, 1021–1026 (2017).
17. Liu, Y., Wilson, T. J. & Lilley, D. M. Structure of a nucleolytic ribozyme that employs a catalytic metal ion. Nat. Chem. Biol. 13, 508–513 (2017).
18. Da Costa, C. P., Okruszek, A. & Sigel, R. K. Controlling ribozyme activity by metal ions. Biochemistry 366, 2910–2917 (2011).
19. Koo, S. C. et al. Transition state features in the hepatitis delta virus ribozyme in solution predicted from molecular simulation. J. Am. Chem. Soc. 138, 3058–3065 (2016).
20. Suslov, N. B. et al. Crystal structure of the Varkud satellite ribozyme. J. Am. Chem. Soc. 133, 486–501 (2011).
21. Lilley, D. M. Catalysis by the nucleolytic ribozymes. Biochem. Soc. Trans. 39, 641–646 (2011).
22. Breaker, R. R. et al. A common speed limit for RNA-cleaving ribozymes and deoxyribozymes. RNA 9, 949–957 (2003).
23. Adams, P. D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D Biol. Crystallogr. 58, 1948–1954 (2002).
24. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501 (2010).
25. Wang, Q. S. et al. The macromolecular crystallography beamline of SSRF. Nucl. Sci. Tech. 26, 12–17 (2015).

Acknowledgements

We thank the staff of the BL-17B and BL-19U1 beamlines at the National Facility for Protein Sciences Shanghai (NFPS) and BL-17U1 at SSRF. We thank Hong Wu and Kaiyi Huang of the Life Sciences Institute (LSI), Zhejiang University for their help in some crystalization solution preparations. The research was supported by grants from the National Science Foundation of China (91640104 and 31670826), the Fundamental Research Funds for the Central Universities (2017QN81010), the new faculty start-up funds from Zhejiang University and the Thousand Young Talents Plan of China (A.R.), by Austrian Science Fund FWF P27947 (R.M.), and by NIH U19CA179564 (DP) and NIH P01CA080748 (Cancer Center Core grant to Memorial Sloan-Kettering Cancer Center).

Author contributions
L.Z. undertook all of the crystallographic experiments with the assistance of Y.Z. under financial interests.

Supplementary Information

L.Z. undertook all of the crystallographic experiments with the assistance of Y.Z. under financial interests.

Additional information

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.