

**Supplementary Figure 1** 

Characterization of different covariation statistics on a positive testset of 104 RNAs.

(a) Plots of the F measure---the harmonic mean of sensitivity (SEN) and positive predictive value (PPV), F=2\*SEN\*PPV / (SEN+PPV)---for four different covariation statistics as a function of the score's E-value, over all alignments, using R=scape with default parameters. (b) Effect of alignment gaps on the different covariation statistics, seen by including all alignment columns (right) as compared to the R-scape default (left). (c) Effect of measuring covariation using a binary classification (whether a pair is canonical Watson-Crick/G:U or not) versus using the full sixteen-way classification. (d) Covariation detection as a function of the number of sequences in the alignments. (e) The F measure for each of the 104 RNA Rfam alignments in the positive testset as a function of average percentage identity, at an E-value threshold of 0.05.



**Supplementary Figure 2** 

Comparison of R-scape to related methods CoMap and MICA [12] on the testset of 104 RNAs.

(a) Sensitivity (percentage of significant base pairs) and positive predictive value (percentage of significant pairs that are base pairs) as a function of the score's E-value. (b) Running times for the three methods (R-scape in black, CoMap cyan, MICA red) on a log-log plot as a function of the number of sequences in the alignment (left) and as a function of the alignment length (right). Running times are for a single 3GHz intel Core i7 with 8GB 1600GHz DDR3 RAM. Running times for R-scape and CoMap include the cost of generating a phylogenetic tree using FastTree [26].



Examples of RNAs with significant covariation support for their proposed structures.

(a) R-scape analysis of a multiple sequence alignment of  $\alpha$ r14, a putative regulatory small RNA in  $\alpha$ -proteobacteria [20,42]. (b) R-cape analysis of a multiple sequence alignment of Arisong RNA, a noncoding RNA identified in the ciliate *Oxytricha* [41]. (c) Example of detecting an underannotated structure, an S15 mRNA leader in  $\gamma$ -proteobacteria that autoregulates ribosomal protein synthesis [19]. Three out of the seven significantly covarying pairs are not in the proposed structure. These covarying pairs support the existence of a conserved pseudoknot, which was already known, but happened to not be annotated in the provided alignment [19]. (d) Example of using R-scape to improve a structural annotation for the Rfam seed alignment for SAM-I riboswitch. The R-scape modified structure has seven significant pairs not included in the Rfam-annotated SAM-I structure. The R-scape structure is in agreement with the secondary structure derived from the SAM-I riboswitch crystal structure (RK Montange & RT Batey, *Nature*, {\bf 441}**441**, 1172-1175, 2006). Notation is as in Figure 2.

## **HOTAIR putative Helix 7**

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Ponge_skelii	1000-0000	UCCOCLUCUC	-100	100A-11	NOO	ACOO.	
Pan_troglodytes	3-060-0606	CU000461UC00	-160	1968-18	IGG	A006	
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Callithriz jacobas	1000-0000	UCCOCLUCIC	-300	NOGA-11	100A	AC00	
Mococa mulotta	3-068-0686	000000000000	-100	IGGA-11	GG1	A006	
Myotis lucifugas	AG-U-0000	CU000668800000	-960	IGGA-11	IGG \	A006	
Lopodenta africana	ACCUSE -COURSE	COCCCUUDOC	-000	MARL-UR	100A	ACOD	
Nicrocobus marines	A003-CC00	UCCOC UCCC	-100	100A-11	100A	ACOO.	
Dipodenys_ordii	A0+C-9CE0	UCCOCCULOCC		NOGA IT	100.	000	
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HOTAIR putative Helix 10

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	and the set of and	3 apparent comparentatory pairs

#### **Supplementary Figure 4**

Covariation analysis of HOTAIR putative helices H7 and H10.

The structural alignments have been extracted from the HOTAIR Domain1 alignment (with 37 sequences) provided in [13]. The H7 and H10 alignments have 28 and 27 sequences respectively, after removing species for which the region does not include any residues. For any two base paired positions, changes are annotated in color relative to the most frequent Watson-Crick or G:U pair. Green arrows indicate the base pairs (one for H7 and 3 for H10) proposed as covarying in [13]. For putative helix H7, the proposed covarying pair (columns 8:36 marked in green) has covariation score -0.16 (E-value 7.74). Gray arrows indicate the best scoring putative Watson-Crick pair (columns 10:30, with a consensus C:G) which was not part of the proposed structure. This best scoring alternative pair would have one U:A compensatory and one U:G half-compensatory changes, and covariation score 3.66 (E-value 5.52). For both alignments, we also provide the R-scape analysis for all pairs. For putative helix H10, the one covariation above the null hypothesis corresponds to a G:G/U:C non-Watson-Crick covariation in a pair of adjacent columns that are not in the proposed structure and are too close to be a base pair.



### ncSRA putative helices H3 and H4

#### Supplementary Figure 5

Covariation analysis of putative helices H3 and H4 of ncSRA.

Color annotation as in Supplementary Figure 4. Green arrows indicate the seven base pairs identified in [21] as significantly covarying. We also provide the R-scape analysis for all pairs in this partial ncSRA alignment.

# ncSRA putative helices H19-H21

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Green_monkey	AAAAGAGAGAGUGUUUU	LGAGELOOCASCCANTO	SANGA GAANDEUGCA DECINCI	GCOGNERACCM	CAR CAULAR CARGE UCCARE CAGAGE UCCAULA
Marmosot Semigral member	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	MANGEMOCASCCANTE	IAAGAGIAADEDIKCASECACI	COULD BARRAGE AND CAR	TACCAULT CONCEPTION CAPTAGE CAPTER
Bushbaby	AAAAAAAdaCICTOLC.	AGA4GAGBC CADU	INAMENDA PROVIDENCE STREET	SCUGAGEMGAACCM	ALCONTACCA OCTUCCA SCA OCCUCCATERA
Chinese_tree_shrew	AAAAAAA GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG	MASCONCCANTE	INAGA BRAAD CUBCA ACTIVES	CONTRACTOR CONTRACTOR	SSCENARCENCERUNCEAUNOSCICENTAL
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Prairie_vele	AAAAGA <b>GAGUGUGUGU</b> UUG	LGA <b>GELOG</b> CCAALO	SANGAGACIN <b>O PUNCIA</b> RIO O CI	CCDGAGAACCAA	SACAGUAC CHORE FUCCA ACAGUEUU CAUNA
Chinese_hamster Golden hamster	AAAAAAAGAGAGAGUGUGUGUGUGUGUGUGUGUGUGUGU	ROMOGRAD COARD	INVERSION OF CONCERNMENT	CCOMMENNCCM	AFERODAE UCCACUUCA- UNGUEUUCAIAA DAERUTAE UUCCACUUCA- CAGOCUUCAIAA
Nouse	AAAAGAAGAAGAGUGUROCUUC	RGAGEAGE CCAAR	SIAGA GIAM <mark>UTUNC<b>A</b> SU</mark> OGAL	CCDGAGAACCAA	SACAAUACCAGGETUCCAACAGOGAUGNIAA
Rot Maked mole-rot	AAAAGAGAGAGOGIROGJUC	RGMGAGAGARCAARS	INACAGA CADEUNCA EDGOCI	CCDGAGAACCAA	GAC NAVAR CHARGE FUCCE ACCEMPTUL OVAN
Guines_pig	AAAAAA GAGAGURACO DIG	LOUSSAND SACAANS	SANGA GANA <mark>U CUGAR DO</mark> NOCI	SCOGNERUCCM	SACCINUAC CACOCITUE DEAGODUCE VIAA
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Cow	ULALGAGEAGOCIGODOIC	LGAGELOG DECCANTO	SANGA GAUNIPERNON DECINES	SCDGASCAGAACCAJ	ANEUGUNE CAGOCAUCCASCAUGACCUGUSA
Domestic_goat	ULALGAGEAGUEUGUUUC	LGAGELODO GECCANTO	SANGA GRAN <b>UC CINCLED</b> CINCU	SCDGASCAGAACCA	AN EUGUAE CAGOGAUCCA SCAUCA CODOUSA
Horse	AAAAGAGAGAGOQUGUQUUG	RGMGR GACCAGE	SANGA GAAN <b>U CUWGA SO</b> ONO	COGNERNOCACCIA	SACCOUNCERGORAUCEA-CROPEUCEAUSA
Cat	AAAAGA4GA4GA4GUGUGUGUGUG	ROUGERACE DECCANES	INALIAN PENGERCAS	SCDGASGAGAAACCAA	ANTEGRATIC CALCENDING CONTRACTOR OF CONTRACT
Dog	AAAAAAA GALCEVENE JIC	NONCONCENSION	INVERSION CONCERNMENT	SCUGASEADDUCCA	ACAUACCAO COUCCAADAGO COCCODAA
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Mallaby	AAADDA <b>GAGCCUDCC</b> JJC	LOODIAA CUOCUI	AND SAERANDEEDACUAEDACU		SAROUSCUCACOCCUOUCUACAACUCCULA
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Covariation analysis of putative helices H19, H20, and H21 of ncSRA.

Color annotation as in Supplementary Figure 4. Green arrows indicate eight base pairs identified in [21] as significantly covarying. We also provide the R-scape analysis for all pairs in this partial ncSRA alignment.



Apparent covariations in 13 aligned Xist RepA region sequences [23].

(a) An alignment column pair was counted as covarying in [23] if it is entirely consistent with Watson-Crick or G:U base pairing, and at least one substitution and no more than two gaps are observed in each column. The dot plot shows 541 column pairs that satisfy these criteria in the RepA alignment used in [23], including (in blue) three of the four cited as support for the secondary structure in [30] (the other has a A:A non canonical pair, thus does not strictly satisfy the rule), 454 pairs that consist of a U+C column and a G+A column (red), and 84 other pairs (black). (b) Example of how single substitutions in conserved U+C and G+A columns can create apparent covariation

HOTA IR Domain1[13] (provided by authors)1493774 (71)526 (792)HOTA IR Domain2[13] (provided by authors)1433174 (73)515 (794)HOTA IR Domain3[13] (provided by authors)1253468 (69)468 (574)
HOTATR Domain1 [13] (provided by authors) 149 37 74 (71) 526 (792)   HOTATR Domain2 [13] (provided by authors) 143 31 74 (73) 515 (794)   HOTATR Domain3 [13] (provided by authors) 125 34 68 (69) 468 (571)
HOTAIR Domain2 [13] (provided by authors) 143 31 74 (73) 515 (794) HOTAIR Domain3 [13] (provided by authors) 125 34 68 (69) 468 (571)
HOTAIR Domain 3 [13] (provided by authors) 125 34 68 (68) 468 (571)
HOTAIR Domain4 [13] (provided by authors) 165 31 69 (68) 637 (884)
SRA ncRNA   similar to [21]   234   76   78 (77)   887 (1181)
XIST   RepA   Structure0   derived from [22]   50 (53)   10   82 (77)   420 (560)
XIST   RepA   Structure1   derived from [22]   74 (90)   10   82 (77)   420 (560)
XIST   RepA   Structure2   derived from [22]   69 (72)   10   82 (77)   420 (560)
XIST   RepA   Structure3   derived from [22]   79 (83)   10   82 (77)   420 (560)
XIST   RepA Targeted Structure-Seq   derived from [23]   88 (99)   13   76 (75)   442 (481)
Arisong RNA [41] (updated from authors) 20 95 66 (65) 65 (150)
α r45 [20,42] (updated from authors) 52 31 87 (86) 180 (186)
αr35 [20,42] (updated from authors) 45 5 78 144 (146)
αr15 [20,42] (updated from authors) 29 51 73 (71) 112 (129)
αr14 [20,42] (updated from authors) 31 98 62 (61) 123 (153)
αr9 [20,42] (updated from authors) 40 26 78 (76) 146 (171)
αr7 [20,42] (updated from authors) 35 26 65 (64) 144 (168)
L1RNA [19] 9 (15) 703 57 (54) 31 (72)
L 10R NA [19] 16 (59) 805 49 (46) 78 (319)
L 20RNA [19] 34 150 65 (63) 87 (127)
L4RNA [19] 59 172 61 (58) 197 (328)
S15RNA [19] 7 166 68 (67) 81 (109)
S1RNA [19] 24 197 61 (60) 117 (179)
S2RNA [19] 17 (45) 614 47 (43) 96 (279)
S4RNA [19] 10 178 74 (73) 110 (129)
S7RNA [19] 33 (34) 158 812 (79) 104 (179)
S8RNA [19] 30 167 82 105 (108)
tRNA RE00005 [15] 21 954 45 (44) 71 (118)
RNase P RNA Bacterial RE00010 [15] 102 458 60 (58) 367 (996)
Purine Riboswitch RE00167 [15] 22 133 55 102 (113)
SAM-I Riboswitch RE00162 [15] 27 433 64 (63) 108 (231)
hAT-Charlie DNA transposon DE0000021 [43] 0 2,000 28 (38) 181 (16 796)
Alu-related SINE/outative ncRNA DE0000073 [43] 0 456 84 (83) 133 (403)
Long Terminal Repeat of retrovirus HERV1 DE0000167[43] 0 13 85 (84) 520 (562)

Properties of the structural alignments used in this study.

The alignments we analyzed are derived from the original alignments such that columns with less than 50% occupied positions are not considered. Information for the original alignments is given in parentheses if different from the analyzed alignment. Alignments are available as Stockholm files in the online Supplementary Information.