Scans of the *MYC* mRNA reveal multiple stable secondary structures—including a 3′ UTR motif, conserved across vertebrates, that can affect gene expression.

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Abstract

The *MYC* gene encodes a human transcription factor and proto-oncogene that is dysregulated in over half of all known cancers. To better understand potential post-transcriptional regulatory features affecting *MYC* expression, we analyzed secondary structure in the *MYC* mRNA using a program that is optimized for finding small locally−folded motifs with a high propensity for function. This was accomplished by calculating folding metrics across the *MYC* sequence using a sliding analysis window and generating unique consensus base pairing models weighted by their lower-than-random predicted folding energy. A series of 30 motifs were identified, primarily in the 5' and 3' untranslated regions, which show evidence of structural conservation and compensating mutations across vertebrate *MYC* homologs. This analysis was able to recapitulate known elements found within an internal ribosomal entry site, as well as discover a novel element in the 3' UTR that is unusually stable and conserved. This novel motif was shown

27   to affect *MYC* expression: likely via modulation of miRNA target accessibility. In addition to

28   providing basic insights into mechanisms that regulate *MYC* expression, this study provides

29   numerous, potentially druggable RNA targets for the *MYC* gene, which is considered

30   "undruggable" at the protein level.

31   Introduction

32   The *MYC* proto-oncogene is an important transcription factor that is required for programmed

33   cell death (apoptosis) and cell proliferation [1]. It is a key component of oncogenesis [2] and,

34   indeed, *MYC* is dysregulated in >50% of all cancers [3]. Post-transcriptional control plays

35   significant roles in the regulation of many genes including *MYC*. Within the 5' untranslated

36   region (UTR) of the *MYC* mRNA is a structured internal ribosomal entry site (IRES) that

37   stimulates cap-independent translation under conditions where cap-dependent translation is

38   inhibited: e.g. during apoptosis [4]. Consistent with other IRESs [5] the *MYC* IRES secondary

39   structure, deduced from *in vitro* chemical probing data [6], is complex and contains two

40   pseudoknots—motifs comprised of "non-nested" base pairing between looped out regions of

41   RNA [7]. In addition to the IRES, other post-transcriptional regulatory mechanisms affect MYC

42   expression—e.g. microRNAs (miRs) [8]—that may be affected by RNA structure [9].

43   To determine if other structured RNA regulatory elements can be playing roles in *MYC*

44   expression, we applied a methodological pipeline for RNA motif discovery that was optimized

45   from studies of the *Xist* lncRNA [10], as well as the Human [11], Zika and HIV genomes [12].

46   There are two major steps in this pipeline: (1) a scanning step, where the RNA is examined

47   using a sliding analysis window to record predicted metrics important for analyzing RNA

48   secondary structure (e.g. the thermodynamic stability); and, (2) an analysis step where unique

49   local motifs are defined then evaluated vs. comparative sequence/structure and/or experimental

50   probing data. Each step is achieved using the programs `ScanFold-Scan` and `ScanFold-`

51   `Fold`, respectively. Used together these programs define the potential RNA structural properties

52   of long sequences and identify motifs likely to be ordered to form, presumably functional,

53   defined structures. This is accomplished by generating consensus structure models across all

54   scanning windows, where base pairs are weighted by their thermodynamic z-score: a measure

55   of the unusual stability of a sequence that is calculated by comparison to the folding energy of

56   matched randomized control sequences. Here, negative values indicate sequences that are

57   ordered to fold and that may be functional [13]. While the primary goal is to deduce what

58   nucleotides may be functionally significant, `ScanFold-Fold` models can also increase

59   prediction accuracy [12]

60   In this report, `ScanFold-Scan` and `ScanFold-Fold` were applied to the longest *MYC* RefSeq

61   mRNA isoform to generate a map of its folding landscape as well as deduce motifs important to

62   the regulation of expression. Numerous motifs were deduced, including those that recapitulated

63   known structures in the *MYC* IRES.

Results

65   `ScanFold-Scan` *mapping of secondary structure in the MYC mRNA*

66   To predict RNA secondary structural characteristics important to *MYC* function, the RefSeq

67   mRNA (NM_001354870) was analyzed using the program `ScanFold-Scan` [12]. This isoform

68   was selected for analysis, as it would contain all potential structural elements found in other

69   (shorter) *MYC* isoforms. The mRNA sequence was analyzed using a 1 nt step and 70 nt window

70   size (Figure 1 and Document S1). Several folding metrics were calculated across analysis

71   windows, which are described in detail in the Materials and Methods and in reference [12].

72   Briefly, the $\Delta G°$ measures the minimum (lowest or most stable) predicted change in the Gibb's

73   free energy upon RNA folding and indicates the thermodynamic stability of RNA structure. The

74   ensemble diversity (ED) is a measure of the structural diversity predicted in the folding

75 ensemble: low numbers indicate one or few dominant structures, while higher numbers indicate

76 multiple conformations or a lack of structure. The z-score measures the propensity of a

77 sequence to be ordered to fold into stable structures. Negative z-scores give the number of

78 standard deviations more thermodynamically stable a sequence is vs. random (see Eq. 1).

79 The global trends in each metric are shown at the top of Figure 1. The trend in the predicted

80 thermodynamic stability approximately follows the GC% and decreases across *MYC*: going from

81 the highly stable 5' UTR to the relatively unstable 3' UTR. This trend is also discernible in the

82 distributions of predicted ΔGs for windows spanning the 5' UTR, coding region and 3' UTR of

83 the mRNA (Fig. S1). The windows spanning the 5' and 3' UTR junctions are less stable than

84 flanking sequences. ED values are more evenly distributed across *MYC*, however, jumps in

85 values for windows spanning the 5' and 3' UTR coding region junctions were observed (Figs. 1

86 and S1). Thermodynamic z-scores ranged from highly negative (-5.0; or 5 standard deviations

87 more stable than random) values to positive ones (+2.8). The average z-score across *MYC* was

88 only slightly negative (-0.4) and there was no evidence of global bias in z-score toward negative

89 values. Notably, windows spanning the 5' UTR junction were shifted toward *positive* z-scores

90 (average of +1.4). These trends in RNA structural stability are more striking when considering

91 "short" UTR isoforms for *MYC* (Fig. S2), which end just upstream of the *MYC* IRES and

92 downstream of Motif 17 (Fig. 1).

93 *`ScanFold-Fold` prediction of functional RNA structural motifs*

94 To deduce local RNA folding that may be functionally significant, all `ScanFold-Scan` prediction

95 windows were analyzed using `ScanFold-Fold`. The `ScanFold-Fold` program generates

96 weighted consensus secondary structures, where minimum free energy (MFE) base pairs that

97 contribute to low z-scores are deduced across the scans. Using a cutoff of -1 `ScanFold-Fold`

98 identified 354 bp (Document S2) across the mRNA, while a cutoff of -2 yields 46 bp that are

99   localized to the 3' UTR. Refolding the mRNA with -1 `ScanFold-Fold` bp as constraints added

100   153 bp to the discovered motifs: e.g. by extending helices or closing unpaired bases in the

101   consensus prediction (Document S2). These 507 bp are divided into 30 motifs that span the

102   *MYC* mRNA (Fig. 1). Motif locations, as expected, correspond to negative dips in z-score;

103   however, dips in ΔG° and ED are also observed at motif sites. The most prominent regions with

104   dips in metrics occur at Motifs 17 and 18 (Fig. 1), which contain very low z-score base pairs

105   (cutoff < -2) deduced by `ScanFold-Fold`. These two motifs, particularly Motif 17, had the most

106   favorable `ScanFold` metrics of any region/motif predicted for MYC.

107   All motif bp were analyzed versus an alignment of 15 vertebrate mRNA sequences (Document

108   S3). Motif 17 had the highest conservation of structure and was supported by the greatest

109   number of consistent and compensatory mutations (Fig. 1). In general, Motifs 7–19 showed

110   evidence of conservation, however, little conservation data was found outside these regions:

111   particularly downstream of Motif 19, where the long 3' UTR annotated for the human *MYC*

112   RefSeq mRNA is not present in the RefSeq mRNA annotations of other species (Document S3).

113   *Analysis of the MYC 5' and 3' UTRs*

114   Motifs 8 and 9 overlap a previously-studied structural feature of the *MYC* mRNA: the IRES [4,

115   6]. Motif 8 is recapitulated in the *MYC* IRES structure Domain 1; only the base pairs in the

116   hairpin spanning nt 110 to 136 (Fig. S3) are shifted over to allow the formation of pseudoknot

117   helix α. Motif 9 partially overlaps Domain 2, where nt 284 to 299 of Domain 2 are refolded into

118   two hairpins (Fig. S3). Structure models were compared vs. an alignment of 50 vertebrate *MYC*

119   UTR sequences (Document S4). The alternative models for Doman 2/Motif 9 are roughly

120   equally well supported by comparative data. Both are comprised of base pairs conserved across

121   vertebrates and that show evidence of possible compensatory mutations: e.g. C279–G284 in

122   Domain 2 vs. A307–U334 and G309–C332 in Motif 9 (Fig. S3). Neither model can be discarded

123   based on these data. Nucleotides within Motif 9 were found to be highly reactive to chemicals in

124   the previous *in vitro* analysis of the *MYC* UTR [6], thus their modeling as single stranded RNA.

125   When overlaid on Motif 9, however, only 4 out of 21 modification sites were inconsistent with the

126   `ScanFold-Fold` generated model (Fig. S3); additionally, sites of AMV reverse transcriptase

127   pausing suggest that this region is structured.

128   Across the *MYC* mRNA, predicted structural metrics are most favorable in the windows that

129   overlap Motif 17 in the 3' UTR (Fig. 1). There are marked dips in the ΔG°, ED and z-score; all

130   indicating importance of structure in this region. The `ScanFold-Fold` predicted base pairs in

131   Motif 17 are also the best-conserved across the 15 vertebrate alignment. Previous work on

132   post-transcriptional regulation of *MYC* found that inclusion of the short 3' UTR sequence led to

133   repression of luciferase expression [14] due to the inclusion of a miR-34 binding site. To

134   determine if RNA structural features in the short 3' UTR (beyond Motif 17) could be playing

135   additional roles, the entire sequence was refolded while constraining Motif 17 base pairs. The

136   resulting global short UTR model (Fig. 2) places the `ScanFold-Fold` predicted Motif 17 into a

137   multibranch loop structure that includes a novel short hairpin. An additional hairpin is also

138   predicted downstream of the multibranch loop. The short 3' UTR model was analyzed vs. an

139   alignment of 59 vertebrate *MYC* 3' UTR sequences (Document S5). This found the highest

140   levels of base pair conservation in the two long Motif 17 hairpins (92% conservation), while the

141   remaining structures are not well-conserved (64% conservation of base pairing). When

142   mutations occur in the highly-conserved Motif 17 they preserve base pairing: e.g. four

143   compensatory (double point) mutations are found in each hairpin in addition to four and two

144   consistent (single point) mutations, respectively (Fig. 2). To see if an orthogonal approach would

145   confirm the 3' UTR model structure or, perhaps, yield a better-conserved alternative model, the

146   program RNAalifold [15] was used to evaluate the short 3' UTR alignment without any base

147   pairing constraints. The RNAalifold program considers both the folding energy and comparative

148   sequence data (implicitly) in prediction; the resulting consensus model (Figure S5) predicts

149 conserved structures that correspond to the two highly-conserved Motif 17 hairpins predicted by

150 `ScanFold-Fold`.

151 *Functional analyses of the MYC 3' UTR*

152 As the most significant motifs predicted in *MYC* occurred in the 3' UTR, a known site of miRNA

153 targeting, the locations of *MYC*-targeting miRNA binding sites were queried vs. predictions of

154 structure. Of nine miRNAs with known interaction sites [8, 14, 16-20], *seven* occurred within

155 Motif 17 (Fig. 3A and B). miR-34a/b/c, miR-449c and let-7a have overlapping seed binding sites

156 in the unstructured region between the two highly-conserved Motif 17 hairpins (Fig. 3A and B).

157 miR-145 binds downstream and partially overlaps the second hairpin. miR-148 has a seed

158 binding site on the terminal stem-loop of the second hairpin. Interestingly, the two miRNAs that

159 bind outside Motif 17 also do so in other `ScanFold-Fold` predicted structural motifs: miR-24

160 binds in the stem region of Motif 18 (Fig. 3C), while miR-185 binds toward the 5' end of Motif 15

161 (Fig. 3D). In all cases, conserved RNA structures are predicted to partially occlude miRNA

162 target binding, potentially modulating their effects.

163 Motif 17 was selected for additional experimental analysis due to it having the strongest

164 `ScanFold` prediction metrics (Fig. 1), high level of structure conservation (Fig. 2), and the

165 presence of multiple miRNA binding sites (Fig. 3A and B). To assess the potential gene

166 regulatory roles of this motif, a luciferase reporter construct was generated incorporating Motif

167 17, along with 27 nt upstream and 11 nt downstream (including a poly(U) tract; Fig. 2). This

168 sequence was inserted into the 3' UTR of the Renilla luciferase (RL) expressing pIS2 vector

169 (referred to as the pIS2-M17 [Motif 17] vector; detailed in the Experimental Procedures). When

170 assayed, the pIS2-M17 vector showed a significant decrease in both relative response ratio

171 (RRR; Fig. 4)—a measure of luciferase activity—and translational efficiency (TE) when

172 compared to the unregulated pIS2 control: a ~24% and ~68% decrease in RRR and TE,

173 respectively. This is consistent with previous analyses of the MYC 3′ UTR, where the entire

174   short UTR isoform was incorporated into pLSV (an analogous Luciferase vector) and, using a

175   similar analysis pipeline, was shown to lead to gene repression [14]. Similarly, ablation of the

176   miR-34a-c seed (and also, seed regions for miRs 449c and let-7a) showed that miRNA targeting

177   was responsible for the repressive effects of this region.

178   To determine if RNA structure present in Motif 17 influences miRNA binding/repression, two

179   mutant constructs, pIS2-AS1 (ablate stem 1) and pIS2-LS1 (lock stem 1), were designed to

180   increase or decrease miRNA site accessibility respectively (Fig. 4) according to the ΔΔG metric

181   of Kurtesz et. al [21]. This metric accounts for both the energy needed to break native mRNA

182   secondary structure and the energy gained by miRNA binding, and it was used to predict

183   miRNA site accessibility for the WT and mutant constructs. The WT sequence, pIS2-M17, has a

184   predicted ΔΔG of -4.67, whereas pIS2-AS1 and pIS2-LS1 have values of -13.56 (more

185   accessible) and +2.55 (less accessible) respectively. When assayed, pIS2-AS1 shows (Fig. 4) a

186   ~11% decrease in its RRR and an increase in TE of ~20% compared to pIS2-M17, however,

187   these values are not significantly different from each other (Fig. 4). pIS2-LS1 showed a

188   substantial increase in both RRR and TE (~68% and ~70% increase respectively) when

189   compared to pIS2-M17.

Discussion

190

191   The analyses performed in this report provide insights into the functions of RNA secondary

192   structure in expression of *MYC*. The `ScanFold-Scan` results map out global features of RNA

193   structure across the *MYC* mRNA. Interesting trends are observed moving across the sequence,

194   where RNA structure thermodynamic stability decreases going 5' to 3', with marked "jumps" in

195   instability observed at the UTR/coding-region junctions (Figs. S1 and S2). Likewise shifts toward

196   more positive ED and z-score values were observed in junction-spanning windows: most

197   dramatically at the 5' junction, which includes both the CUG (non-canonical) and AUG

198   (canonical) translation initiation sites. These results indicate a lack of stable structure here,

199   reiterating previous observations that indicate inhibitory roles for thermodynamically stable RNA

200   secondary structure at initiation sites [22]. We additionally find evidence that evolution may be

201   specifically selecting for *MYC* initiation site sequences that are ordered to be less-stable than

202   that predicted for sequences of similar composition (thus the positive z-scores); as well, the

203   junction sequence is expected to have a volatile conformational ensemble, where no particular

204   structure dominates (high ED).

205   The high and low respective thermodynamic stabilities of the 5' and 3' UTRs (Figs. S1 and S2)

206   indicate differing roles for RNA folding in these regions. The highly-stable 5' UTR would be

207   expected to inhibit canonical translation by obstructing scanning ribosomes; thus, the presence

208   of an IRES in the *MYC* mRNA. This can provide mechanisms for fine-tuning the post-

209   transcriptional regulation of the *MYC* gene by allowing it to be translated in a cap-independent

210   manner. The *MYC* IRES was shown to be active in some, but not all tissue types and the

211   variability of activity is attributed to the presence, or lack of, trans-regulatory elements (e.g.

212   RBPs; [4]). This demonstrates how cis-elements of the mRNA can interact with trans-regulatory

213   elements to diversify (i.e. regulate) the cellular levels of a protein.

214   In contrast, the low stability of the 3' UTR suggests a need for increased accessibility of the

215   mRNA sequence: e.g. for intermolecular interactions with post-transcriptional regulatory factors

216   such as miRNAs and regulatory proteins. Counterintuitively, the sites with the greatest evidence

217   of having been ordered to fold into a specific structure are in the 3' UTR (e.g. Motifs 17 and 18

218   in Fig. 1). Motif 17, for example, is the most well-conserved structured region in *MYC*—even

219   more so than the IRES domain (Figs. 2 and S3)—and is supported via multiple compensatory

220   and consistent base mutations. The highly favorable metrics and deep conservation of this motif

221   throughout vertebrates indicated its biological importance, which was borne out by the analysis

222   of miRNA binding sites (Fig. 3A and B) and Motif 17 function (Fig. 4). This motif acts as a hub

223   for miRNA interactions and may organize the *MYC* miRNA target sites for interactions: e.g. the

224   two highly-conserved hairpins may act as a "structure cassette" for maintaining the single-

225   strandedness of the miR-34, -449c, and -let-7a seed binding regions, while modulating the

226   accessibility of additional bases for miRNA pairing that can affect the outcome of miRNA

227   targeting: e.g. translational repression.

228   The second most favorable motif (Motif 18; Fig. 1) contains a miR-24 interacting region (Fig.

229   3C). Notably, this interaction is "seedless" [17]—only three of the miR-24 seed nt are base

230   paired to *MYC* (Fig. 3C). Most of the miR-24-interacting nt on *MYC* are predicted to be bound

231   up in structure. Here, as in other interaction sites, RNA folding may be modulating accessibility.

232   We observed that reducing the accessibility of miRNA binding in Motif 17 via pIS2-LS1

233   increased RRR and TE—potentially by reducing the amounts of miRNA-mediated gene

234   repression. On the other hand, pIS2-M17 (the WT motif) and pIS2-AS1 (that ablates the first

235   stem loop) had similar RRR and TE. Additional cellular factors may influence how these

236   structures affect miRNA targeting: e.g. interactions with RNA binding proteins or post-

237   transcriptional modifications can affect RNA folding. Thus, structural motifs and their additional

238   interactions and alterations may be a way to fine tune the effects of miRNA regulation of *MYC*.

239   Conserved RNA structural motifs may also serve other functions in regulating *MYC* expression.

240   Both Motif 17 and 18 occur in a particularly GC-poor and thermodynamically unstable region of

241   the 3' UTR (Fig. 1). This is due to long stretches of highly-conserved poly(U) (u)ridine) tracts that

242   occur between and around these structural motifs (Documents S3 and S5). Interestingly,

243   poly(U) tracts have been found to stabilize mRNA sequences via structural interactions with the

244   poly(A) (a)denosine) tail [23]. Thus, an additional function of *MYC* 3' UTR structural motifs may

245   be to organize poly(U) tracts to facilitate interactions with the poly(A) tail. For example, in Figure

246   2 the poly(U) sequence is bulged out between the second Motif 17 hairpin and the basal stem of

247   the predicted multibranch loop, which could allow the poly(A) tail to "wind" around this structure:

248   e.g. in an analogous way to poly(U)-poly(A) interactions in viral and ncRNA stabilizing elements

249   [24, 25]. Notably, our qPCR data does not indicate degradation of miRNA targeted RL

250   transcripts. Instead, slight transcript accumulation is observed in experimental samples (which

251   contain the poly(U) tract) compared to unregulated pIS2 samples (Table S1). Motif 17, in

252   addition to regulating miRNA target accessibility, may be affecting transcript stability by

253   modulating interactions of the poly(U) tract with the poly(A) tail of the mRNA. More investigation

254   is needed to parse out these interactions.

255   Additional functional motifs are predicted beyond Motif 18 (Fig. 1), which may also be

256   functionally significant. Interestingly, cancer-associated *MYC* translocations [26] can lead to

257   UTR truncations that delete predicted motifs: potentially impacting function and contributing to

258   *MYC* dysregulation. Likewise, seven predicted motifs fall within the *MYC* coding region, which

259   may be functionally significant: e.g. by providing roadblocks for translation that can affect protein

260   folding [27] or by affecting interactions with regulatory factors [28]. Notably, miR-185 targets a

261   sequence that overlaps Motif 15 (Fig. 3D), which falls within the *MYC* coding region (Fig. 1).

262   Awareness of the importance of miRNA targeting in coding regions is growing [29] and,

263   presumably, additional *MYC* miRNA interactors remain to be discovered.

264   To conclude, this report provides valuable information on *MYC* mRNA secondary structure that

265   has implications toward a better understanding of post-transcriptional gene regulation. In

266   addition to providing global structural data, discreet local motifs with a high propensity for

267   function are proposed, including a particularly interesting motif in the 3' UTR that has been

268   functionally validated. We showed Motif 17 possesses post-transcriptional regulatory function

269   and, at the very least, this function is a result of structure regulated miRNA targeting. Our

270   findings illustrate the utility of `ScanFold-Scan` and `ScanFold-Fold` in finding structured,

271   regulatory motifs and highlight the important role of RNA secondary structure in the post-

272   transcriptional gene regulation of *MYC* expression. This study provides a roadmap for further

273   analyses of the structure/function relationships in the *MYC* mRNA and a framework for

274   understanding other experimental results. For example, identified clinically significant sequence

275   variants can be cross-referenced to these results to deduce their potential impact on RNA

276   folding. Additionally, these results generate a large list of structural motifs that may be

277   druggable targets [30, 31] for *MYC*, which is considered undruggable at the protein level [3].

278   # Materials and Methods

279   *In silico analyses*

280   The *Homo sapiens MYC* RefSeq mRNA sequence was downloaded from the NCBI nt database

281   (GenBank Accession: NM_002467.5). `ScanFold-Scan` was run using a single nt step size and

282   window sizes of 70 (Document S1) and 120 nt (results with the longer window size were

283   unchanged [data not shown], this 70 nt window was used in subsequent analyses). RNA

284   structural metrics were calculated for windows using the RNAfold algorithm [32] using the

285   Turner energy model [33, 34] at 37 °C. Z-score calculations were performed using the following

286   equation (adapted from the approach of [13]):

287     $$\textit{z-score} = (\Delta G^\circ_{native} - \overline{\Delta G^\circ}_{random})/\sigma \quad \text{(Eq. 1)}$$

288   Here, ΔG°_{native} is the native sequence <u>m</u>inimum <u>f</u>ree <u>e</u>nergy (MFE) predicted by RNAfold.

289     $\overline{\Delta G^\circ}_{random}$ is the average MFE predicted for 100X mononucleotide randomized sequences. The

290   standard deviation, σ, is calculated across all sequences. The other calculated data are: the P-

291   value, which measures the fraction of random sequences that are more stable than native in the

292   z-score calculation (this acts as a quality control measure for the z-score); the MFE ΔG°, which

293   measures the thermodynamic stability of RNA secondary structure formation; the MFE base

294   pairs that generate the MFE ΔG°, which are output in "dot-bracket" notation; the <u>e</u>nsemble

295   <u>d</u>iversity (ED), which provides an estimate of the structural diversity in the RNA conformational

296   ensemble (e.g. low ED indicates a single dominant conformation); the fraction of the (f)MFE in

297   the ensemble, which estimates the contribution of the MFE conformation to the ensemble; the

298   ensemble centroid structure, which is the conformation most similar to others in the ensemble;

299   and the nt frequencies and GC percentages.

300   `ScanFold-Scan` prediction windows were next analyzed using the program `ScanFold-Fold`

301   to deduce consensus motifs weighted by the z-score. The `ScanFold-Fold` method is detailed

302   in [12]. Resulting output consisted of a list of all base pairing partners predicted for each

303   nucleotide of the *MYC* mRNA (Document S6) and a list of the most favorable base pairing

304   partners when weighting by z-score (Document S7). From the latter, base pairs which

305   contributed to consistently negative z-scores (i.e. bps with average z-scores less -1 from

306   Document S7) were used as constraints in an RNAfold prediction on the entire mRNA under the

307   additional constraint of a maximum bp distance of 300 nt. Base pairs that extended `ScanFold-`

308   `Fold` helixes were identified and used to generate the final motif models (Document S2). For

309   visualizing the results of modeling, 2D rendering were generated using VARNA [35] and figures

310   were produced with Adobe Illustrator.

311   For the analysis of conservation of `ScanFold-Fold` motifs across *MYC*, homologous mRNAs

312   for 14 representative vertebrates were obtained from the NCBI RefSeq RNA database [36]. This

313   database was also queried using BLAST [37] to deduce homologs for the "short" *MYC* 5' and 3'

314   UTR sequences. Alignments for the mRNA (Document S3) and UTRs (Documents S4 and S5)

315   were performed using MAFFT [38], implementing the MAFFT-E-INS-i and MAFFT-G-INS-i

316   strategies, respectively [39].

317   A global model for the short 3' UTR (defined/used in a previous study of miRNA targeting [14]

318   was generated by constraining base pairs from Motif 17 and refolding the remaining sequence

319   using RNAfold [32]. A consensus secondary structure for the short *MYC* 3' UTR was predicted

320   (Fig. S3) using RNAalifold [15] with the 3' UTR alignment (Document S5) as input.

321   *Experimental analyses*

322   **Cell Culture.** HeLa cells were incubated at 37°C and 5% CO_2 and maintained in DMEM

323   supplemented with 10% FBS, penicillin and streptomycin, and L-glutamine. Cells were

324   passaged at 60-80% confluence and used between 3-40 passages.

325   **Luciferase Vectors**. For our experiments, two luciferase plasmid vector backbones were used.

326   Both the transfection control vector, pIS0, which encoded firefly (FF) luciferase, and the

327   experimental vector, pIS2, which encoded renilla (RL) luciferase, were gifts from David Bartel

328   (Addgene plasmid # 12178 ; http://n2t.net/addgene:12178 ; RRID:Addgene_12178) and

329   (Addgene plasmid # 12177 ; http://n2t.net/addgene:12177 ; RRID:Addgene_12177). The

330   pcDNA3.1-miR34a vector was a gift from Heidi Schwarzenbach (Addgene plasmid # 78125 ;

331   http://n2t.net/addgene:78125 ; RRID:Addgene_78125).

332   To test the post-transcriptional regulation of `ScanFold-Fold` predicted motifs, the Motif 17

333   sequence, along with 27 nt upstream and 11 nt downstream were incorporated into the 3'-UTR

334   of pIS2 to generate pIS2-M17. Mutants that destabilize, pIS2-AS1, or stabilize, pIS2-LS1, the

335   structure present in pIS2-M17 were generated. For pIS2-AS1, 6 mutations were incorporated

336   that disrupt canonical base pairing in the first conserved hairpin. To generate pIS2-LS1, 3

337   mutations and 1 base deletion were introduced in the bulge on the upstream side of the first

338   conserved hairpin. Mutations that destabilize or stabilize Motif 17 were predicted using the ΔΔG

339   metric as a measure of miRNA site accessibility (Kertesz et al., 2007). 70 nt upstream and 70 nt

340   downstream of the miRNA target site were included in our ΔΔG calculations.

341   The sequences for pIS2-M17, pIS2-AS1, and pIS2-LS1 were ordered as gBlocks from IDT and

342   cloned using AgeI (5') and Spe1 (3') restriction sites (sequences in Supplemental Table S2).

343   Insertion of experimental sequences into the 3'-UTR of pIS2 required double restriction enzyme

344   digest (using AgeI and SpeI from NEB) of both the gBlock and pIS2, following digestion,

345   fragment and vector DNA were purified (Zymo DNA Clean and Concentrator kit), ligated (T4

346   Ligase from ThermoFischer), and transformed into DH5α-T1 competent cells using standard

347   procedures. Carbenicillin selected colonies were cultured and plasmids were extracted (Qiaprep

348   kit) and sequenced using an Applied Biosystems 3730xl DNA Analyzer.

349   **Dual Luciferase Assay.** Dual luciferase assays followed recommendations of an established

350   method (Etten et al., 2013). In brief, the pIS0 vector (FF) is transfected at constant levels across

351   all samples to serve as an internal control to which RL luciferase expression is normalized. All

352   samples were run as biological triplicates. HeLa cells were counted using a hemocytometer and

353   plated in a 24-well dish at a density of 50,000 cells per well. After 48 hours, cells were

354   transfected using Lipofectamine 3000 (ThermoFischer) with 500 nanograms total plasmid DNA

355   at a 1:1:8 ratio (pIS0:pIS2-based:pcDNA3.1-miR34a). Twenty-four hours later, cells were

356   trypsinized, resuspended, and split into each of a 24-well plate for RNA analysis (1 ml) and a

357   96-well plate for the dual luciferase assay (0.2 ml). After another 24-hour incubation, cells in the

358   96-well dish were lysed, and luciferase activity was measured using Promega's Dual Luciferase

359   Reagent Assay kit on a Biotek Synergy 2 plate reader with a collection time of 10 seconds.

360   Relative response ratios (RRR), the ratio of RL to FF relative light units (RLUs), were calculated

361   for each sample and then normalized to the empty, unregulated pIS2 RRR. Cells from the 24-

362   well plate were placed in TRIzol (ThermoFisher) and either stored at -80°C or immediately

363   processed as below.

364   **RNA Processing and qPCR Analysis**. Cellular RNA was purified from samples in TRIzol using

365   Zymo's Direct-Zol RNA Miniprep kit. Purified RNA was then Dnase I treated (NEB) for 2 hours at

366   37°C and the resulting DNase-treated RNA was purified with Zymo's RNA Clean and

367   Concentrator kit. Reverse transcription was done using 1 microgram of purified RNA, random

368   hexamers, and Superscript III (ThermoFisher).

369   Relative abundance of RL transcripts across samples were measured by qPCR, performed

370   using PowerUp SYBR Green Master Mix on 1% cDNA input on an Applied Biosystems

371   QuantStudio 3 instrument (ThermoFisher). Data were analyzed using the ΔΔCt method, where

372   the relative abundance of RL transcripts in the samples were determined using the FF transcript

373   as the reference gene. Translational efficiencies (TE), a normalization metric ($RRR/2^{[-\Delta\Delta Ct_{RL}]}$),

374   were calculated for each sample. Primers used in qPCR were: RL FWD 5'-

375   ggaattataatgcttatctacgtgc-3'; RL REV 5'-cttgcgaaaaatgaagaccttttac-3'; FF FWD 5'-

376   ctcactgagactacatcagc-3'; and FF REV 5'-tccagatccacaaccttcgc-3'.

377   All data are available in the Supplemental Information. `ScanFold-Scan` and `ScanFold-Fold`

378   are available for download from GitHub: https://github.com/moss-lab/`ScanFold`. RNAfold and

379   RNAalifold are both bundled within the ViennaRNA package [32]: available at:

380   https://www.tbi.univie.ac.at/RNA/.

Acknowledgments

Figure Legends

386   **Figure 1.** Summary of `ScanFold-Scan` and `ScanFold-Fold` results for the short UTR *MYC*

387   mRNA. At the top are charts indicating the predicted `ScanFold-Scan` metrics across the

388   mRNA. The bars are set at the 1st nt of the 70 nt window, thus data corresponds to the 70 nt

389   downstream of the bar. Below these is a cartoon of the *MYC* mRNA with UTRs and coding

390   region represented in thin and thick black lines, respectively. This cartoon is annotated with

391   boxes which depict the location and extent of `ScanFold-Fold` predicted motifs shaded red

392   based on the average z-score of windows in which motif base pairs occurred. Below these are

393   RNA secondary structure arc diagrams which depict the most favorable base pairs predicted via

394   `ScanFold-Fold,` colored according the average z-scores of windows in which they appear

395   (with blue, green and yellow corresponding to less than -2, -1 and 0 z-scores averages

396   respectively). Below these, are refolded models of the motifs built with -1 average z-score bps

397   as constraints. Each is annotated with their bp conservation as determined from an alignment of

398   15 representative mRNAs (Document S3) indicated by shading on the base pair (see key).

399   Circled bases are sites of putative structure-preserving consistent and compensatory mutations.

400   **Figure 2.** Short MYC 3' UTR model. HP2–3 comprise Motif 17 of the `ScanFold-Fold` results,

401   which were constrained in the calculation. Base pair conservation shading indicated in the key

402   and data are taken from a comparison of 59 RefSeq mRNA vertebrate alignment (Document

403   S5).

404   **Figure 3.** Annotations of miRNA binding sites on `ScanFold-Fold` predicted motifs. (A) Shows

405   miRNA sequences above the "dot-bracket" structure of Motif 17 (matched brackets indicated

406   base pairs). Seed sites and the complements on Motif 17 are colored. (B) Shows miRNA seed

407   binding sites annotated on the 2D model of Motif 17. (C) Shows base-pairing between miR-24

408   and the 2D model of Motif 18. (C) Shows base-pairing between miR-24 and the 2D model of

409   Motif 15.

410   **Figure 4.** RRRs and TEs were calculated for each set of samples and normalized to the

411   unregulated activity of pIS2. Error bars report the standard error. Experimental vectors pIS2-

412   M17, pIS2-AS1, and pIS2-LS1 all display activity that differs from pIS2. pIS2-M17 and pIS2-AS1

413   both show decreased RRRs while pIS2-LS1, which was designed to have a more stable and

414   less accessible 3'-UTR, shows an increase in RRR compared to pIS2. pIS2-MSC displays a

415   large decrease in TE and pIS2-AS1, which is predicted to have a more accessible miRNA site,

416   displays a TE which is slightly increased compared to pIS2-M17. The TE of pIS2-LS1 is

417   markedly greater than pIS2-M17, possibly reflecting the decrease in miRNA target site

418   accessibility. The ΔΔG values for pIS2-M17, pIS2-AS1, and pIS2-LS1 are also shown along with

419   destabilizing pIS2-AS1 mutations (displayed in red) and stabilizing pIS2-LS1 mutations

420   (displayed in green).

Supplemental Material

422   Supplemental material is available for this report.

References

424   1.   Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol
425   Cell Biol. 1999;19(1):1-11. Epub 1998/12/22. PubMed PMID: 9858526; PubMed Central
426   PMCID: PMCPMC83860.
427   2.   Dang CV. MYC on the path to cancer. Cell. 2012;149(1):22-35. Epub 2012/04/03. doi:
428   10.1016/j.cell.2012.03.003. PubMed PMID: 22464321; PubMed Central PMCID:
429   PMCPMC3345192.
430   3.   Dang CV, Reddy EP, Shokat KM, Soucek L. Drugging the 'undruggable' cancer targets.
431   Nat Rev Cancer. 2017;17(8):502-8. Epub 2017/06/24. doi: 10.1038/nrc.2017.36. PubMed PMID:
432   28643779; PubMed Central PMCID: PMCPMC5945194.
433   4.   Stoneley M, Subkhankulova T, Le Quesne JP, Coldwell MJ, Jopling CL, Belsham GJ, et
434   al. Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the
435   nuclear compartment. Nucleic Acids Res. 2000;28(3):687-94. Epub 2000/01/19. PubMed PMID:
436   10637319; PubMed Central PMCID: PMCPMC102558.
437   5.   Yamamoto H, Unbehaun A, Spahn CMT. Ribosomal Chamber Music: Toward an
438   Understanding of IRES Mechanisms. Trends Biochem Sci. 2017;42(8):655-68. Epub
439   2017/07/08. doi: 10.1016/j.tibs.2017.06.002. PubMed PMID: 28684008.
440   6.   Le Quesne JP, Stoneley M, Fraser GA, Willis AE. Derivation of a structural model for the
441   c-myc IRES. J Mol Biol. 2001;310(1):111-26. Epub 2001/06/23. doi: 10.1006/jmbi.2001.4745.
442   PubMed PMID: 11419940.
443   7.   Liu B, Mathews DH, Turner DH. RNA pseudoknots: folding and finding. F1000 Biol Rep.
444   2010;2:8. doi: 10.3410/B2-8. PubMed PMID: 20495679; PubMed Central PMCID:
445   PMCPMC2873773.
446   8.   Jackstadt R, Hermeking H. MicroRNAs as regulators and mediators of c-MYC function.
447   Biochim Biophys Acta. 2015;1849(5):544-53. Epub 2014/04/15. doi:
448   10.1016/j.bbagrm.2014.04.003. PubMed PMID: 24727092.
449   9.   Wan Y, Kertesz M, Spitale RC, Segal E, Chang HY. Understanding the transcriptome
450   through RNA structure. Nat Rev Genet. 2011;12(9):641-55. doi: 10.1038/nrg3049. PubMed
451   PMID: 21850044; PubMed Central PMCID: PMCPMC3858389.
452   10.   Fang R, Moss WN, Rutenberg-Schoenberg M, Simon MD. Probing Xist RNA Structure in
453   Cells Using Targeted Structure-Seq. PLoS Genet. 2015;11(12):e1005668. doi:

454   10.1371/journal.pgen.1005668. PubMed PMID: 26646615; PubMed Central PMCID:
455   PMCPMC4672913.
456   11.   Andrews RJ, Baber L, Moss WN. RNAStructuromeDB: A genome-wide database for
457   RNA structural inference. Sci Rep. 2017;7(1):17269. doi: 10.1038/s41598-017-17510-y.
458   PubMed PMID: 29222504; PubMed Central PMCID: PMCPMC5722888.
459   12.   Andrews R.J. RJ, Moss W.N. ScanFold: an approach for genome-wide discovery of
460   local RNA structural elements—applications to Zika virus and HIV. PeerJ Preprints.
461   2018;6:e6136. doi: https://doi.org/10.7717/peerj.6136
462   13.   Clote P, Ferre F, Kranakis E, Krizanc D. Structural RNA has lower folding energy than
463   random RNA of the same dinucleotide frequency. RNA. 2005;11(5):578-91. doi:
464   10.1261/rna.7220505. PubMed PMID: 15840812; PubMed Central PMCID: PMCPMC1370746.
465   14.   Kong YW, Cannell IG, de Moor CH, Hill K, Garside PG, Hamilton TL, et al. The
466   mechanism of micro-RNA-mediated translation repression is determined by the promoter of the
467   target gene. Proc Natl Acad Sci U S A. 2008;105(26):8866-71. Epub 2008/06/27. doi:
468   10.1073/pnas.0800650105. PubMed PMID: 18579786; PubMed Central PMCID:
469   PMCPMC2449332.
470   15.   Bernhart SH, Hofacker IL, Will S, Gruber AR, Stadler PF. RNAalifold: improved
471   consensus structure prediction for RNA alignments. BMC Bioinformatics. 2008;9:474. doi:
472   10.1186/1471-2105-9-474. PubMed PMID: 19014431; PubMed Central PMCID:
473   PMCPMC2621365.
474   16.   Luan W, Wang Y, Chen X, Shi Y, Wang J, Zhang J, et al. PKM2 promotes glucose
475   metabolism and cell growth in gliomas through a mechanism involving a let-7a/c-Myc/hnRNPA1
476   feedback loop. Oncotarget. 2015;6(15):13006-18. Epub 2015/05/08. doi:
477   10.18632/oncotarget.3514. PubMed PMID: 25948776; PubMed Central PMCID:
478   PMCPMC4536995.
479   17.   Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, O'Day E, et al. miR-24 Inhibits cell
480   proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3'UTR
481   microRNA recognition elements. Mol Cell. 2009;35(5):610-25. Epub 2009/09/15. doi:
482   10.1016/j.molcel.2009.08.020. PubMed PMID: 19748357; PubMed Central PMCID:
483   PMCPMC2757794.
484   18.   Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S, et al. p53 represses c-Myc through
485   induction of the tumor suppressor miR-145. Proc Natl Acad Sci U S A. 2009;106(9):3207-12.
486   Epub 2009/02/10. doi: 10.1073/pnas.0808042106. PubMed PMID: 19202062; PubMed Central
487   PMCID: PMCPMC2651330.
488   19.   Liao JM, Lu H. Autoregulatory suppression of c-Myc by miR-185-3p. J Biol Chem.
489   2011;286(39):33901-9. Epub 2011/08/13. doi: 10.1074/jbc.M111.262030. PubMed PMID:
490   21832077; PubMed Central PMCID: PMCPMC3190790.
491   20.   Miao LJ, Huang SF, Sun ZT, Gao ZY, Zhang RX, Liu Y, et al. MiR-449c targets c-Myc
492   and inhibits NSCLC cell progression. FEBS Lett. 2013;587(9):1359-65. Epub 2013/03/20. doi:
493   10.1016/j.febslet.2013.03.006. PubMed PMID: 23507140.
494   21.   Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in
495   microRNA target recognition. Nat Genet. 2007;39(10):1278-84. Epub 2007/09/26. doi:
496   10.1038/ng2135. PubMed PMID: 17893677.
497   22.   Gu W, Zhou T, Wilke CO. A universal trend of reduced mRNA stability near the
498   translation-initiation site in prokaryotes and eukaryotes. PLoS Comput Biol.
499   2010;6(2):e1000664. Epub 2010/02/09. doi: 10.1371/journal.pcbi.1000664. PubMed PMID:
500   20140241; PubMed Central PMCID: PMCPMC2816680.
501   23.   Geisberg JV, Moqtaderi Z, Fan X, Ozsolak F, Struhl K. Global analysis of mRNA isoform
502   half-lives reveals stabilizing and destabilizing elements in yeast. Cell. 2014;156(4):812-24. Epub
503   2014/02/18. doi: 10.1016/j.cell.2013.12.026. PubMed PMID: 24529382; PubMed Central
504   PMCID: PMCPMC3939777.

505   24.   Tycowski KT, Shu MD, Borah S, Shi M, Steitz JA. Conservation of a triple-helix-forming
506   RNA stability element in noncoding and genomic RNAs of diverse viruses. Cell Rep.
507   2012;2(1):26-32. Epub 2012/07/31. doi: 10.1016/j.celrep.2012.05.020. PubMed PMID:
508   22840393; PubMed Central PMCID: PMCPMC3430378.
509   25.   Brown JA, Valenstein ML, Yario TA, Tycowski KT, Steitz JA. Formation of triple-helical
510   structures by the 3'-end sequences of MALAT1 and MENbeta noncoding RNAs. Proc Natl Acad
511   Sci U S A. 2012;109(47):19202-7. Epub 2012/11/07. doi: 10.1073/pnas.1217338109. PubMed
512   PMID: 23129630; PubMed Central PMCID: PMCPMC3511071.
513   26.   Ott G, Rosenwald A, Campo E. Understanding MYC-driven aggressive B-cell
514   lymphomas: pathogenesis and classification. Blood. 2013;122(24):3884-91. Epub 2013/09/07.
515   doi: 10.1182/blood-2013-05-498329. PubMed PMID: 24009228.
516   27.   Faure G, Ogurtsov AY, Shabalina SA, Koonin EV. Role of mRNA structure in the control
517   of protein folding. Nucleic Acids Res. 2016;44(22):10898-911. doi: 10.1093/nar/gkw671.
518   PubMed PMID: 27466388; PubMed Central PMCID: PMCPMC5159526.
519   28.   Glisovic T, Bachorik JL, Yong J, Dreyfuss G. RNA-binding proteins and post-
520   transcriptional gene regulation. FEBS Lett. 2008;582(14):1977-86. Epub 2008/03/18. doi:
521   10.1016/j.febslet.2008.03.004. PubMed PMID: 18342629; PubMed Central PMCID:
522   PMCPMC2858862.
523   29.   Zhang K, Zhang X, Cai Z, Zhou J, Cao R, Zhao Y, et al. A novel class of microRNA-
524   recognition elements that function only within open reading frames. Nat Struct Mol Biol.
525   2018;25(11):1019-27. Epub 2018/10/10. doi: 10.1038/s41594-018-0136-3. PubMed PMID:
526   30297778; PubMed Central PMCID: PMCPMC6219929.
527   30.   Tran T, Disney MD. Identifying the preferred RNA motifs and chemotypes that interact
528   by probing millions of combinations. Nat Commun. 2012;3:1125. Epub 2012/10/11. doi:
529   10.1038/ncomms2119. PubMed PMID: 23047683; PubMed Central PMCID: PMCPMC3533436.
530   31.   Angelbello AJ, Chen JL, Childs-Disney JL, Zhang P, Wang ZF, Disney MD. Using
531   Genome Sequence to Enable the Design of Medicines and Chemical Probes. Chem Rev.
532   2018;118(4):1599-663. Epub 2018/01/13. doi: 10.1021/acs.chemrev.7b00504. PubMed PMID:
533   29322778; PubMed Central PMCID: PMCPMC5989578.
534   32.   Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF, et al.
535   ViennaRNA Package 2.0. Algorithms Mol Biol. 2011;6:26. doi: 10.1186/1748-7188-6-26.
536   PubMed PMID: 22115189; PubMed Central PMCID: PMCPMC3319429.
537   33.   Mathews DH, Disney MD, Childs JL, Schroeder SJ, Zuker M, Turner DH. Incorporating
538   chemical modification constraints into a dynamic programming algorithm for prediction of RNA
539   secondary structure. Proc Natl Acad Sci U S A. 2004;101(19):7287-92. doi:
540   10.1073/pnas.0401799101. PubMed PMID: 15123812; PubMed Central PMCID:
541   PMCPMC409911.
542   34.   Turner DH, Mathews DH. NNDB: the nearest neighbor parameter database for
543   predicting stability of nucleic acid secondary structure. Nucleic Acids Res. 2010;38(Database
544   issue):D280-2. Epub 2009/11/03. doi: 10.1093/nar/gkp892. PubMed PMID: 19880381; PubMed
545   Central PMCID: PMCPMC2808915.
546   35.   Darty K, Denise A, Ponty Y. VARNA: Interactive drawing and editing of the RNA
547   secondary structure. Bioinformatics. 2009;25(15):1974-5. doi: 10.1093/bioinformatics/btp250.
548   PubMed PMID: 19398448; PubMed Central PMCID: PMCPMC2712331.
549   36.   O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference
550   sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional
551   annotation. Nucleic Acids Res. 2016;44(D1):D733-45. Epub 2015/11/11. doi:
552   10.1093/nar/gkv1189. PubMed PMID: 26553804; PubMed Central PMCID: PMCPMC4702849.
553   37.   Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool.
554   J Mol Biol. 1990;215(3):403-10. Epub 1990/10/05. doi: 10.1016/S0022-2836(05)80360-2.
555   PubMed PMID: 2231712.

556   38.   Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple
557   sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30(14):3059-66.
558   Epub 2002/07/24. PubMed PMID: 12136088; PubMed Central PMCID: PMCPMC135756.
559   39.   Katoh K, Standley DM. MAFFT: iterative refinement and additional methods. Methods
560   Mol Biol. 2014;1079:131-46. Epub 2013/10/31. doi: 10.1007/978-1-62703-646-7_8. PubMed
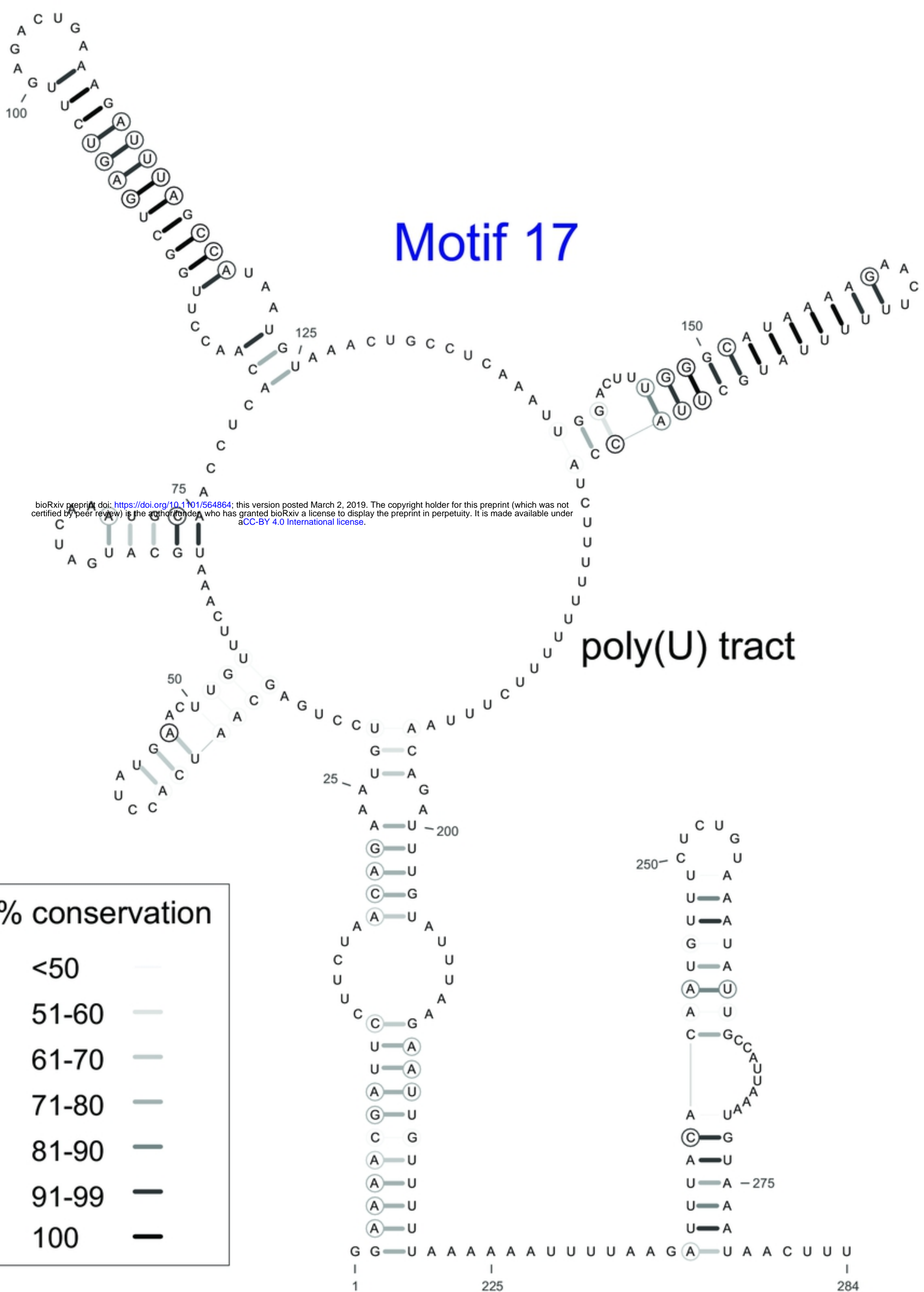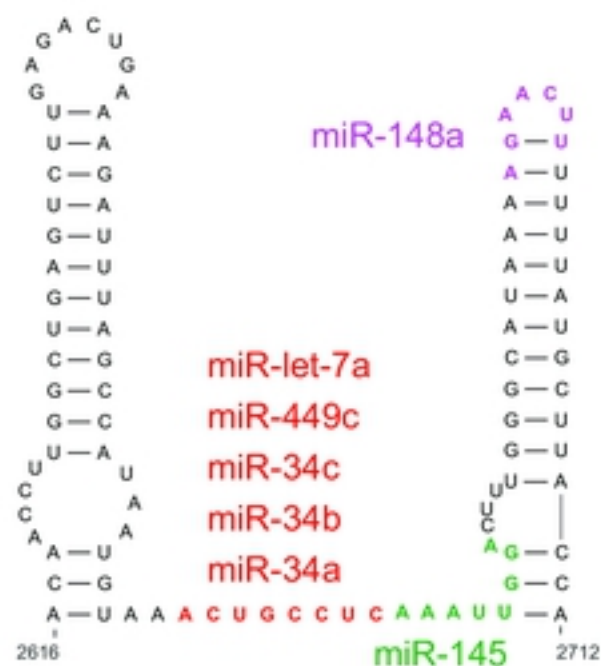561   PMID: 24170399.

562

563

Motif 17

poly(U) tract

% conservation

<50	—
51-60	—
61-70	—
71-80	—
81-90	—
91-99	—
100	—

Figure 2

Figure 3

pIS2-M17
ΔΔG = -4.67 kcal/mol

pIS2-AS1
ΔΔG = -13.56 kcal/mol

pIS2-LS1
ΔΔG = +2.55 kcal/mol

Figure 4

Figure 1